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13. ABSTRACT (Maximum 200 Words)
The purpose of this study is to utilize adaptein libraries coded within pantropic retroviral vectors the confer protection against rickettsial pathogens and to study themolecular pathogenesis of rickettsioses. The following specific aims were proposed: 1) To establish heterogeneous cell populations, with each cell expression a unique member of a complex combinatorial peptide-based (e.g., adaptein) library and challenge with *R. prowazekii, R. rickettsii,* and *O. tsutsugamushi*; 2) To determine the role of NF-kB, cytokines (TNF-a, IFN-g, RANTES), ROS, and NO in intracellular killing of rickettsia-infected monolayers containing adapteins and 3) To characterize signal transduction pathways modulating the cytoskeletal events responsible for the increased vascular permeability. During the second year of the project, we were ale to produce and optimize production of adaptein libraries containing 6-, 12-, and 18-mer peptides and EGFP in he retroviral vector. Successful transfection of rat derived brain microvascular endothelial cells and human brain primary microvascular endothelial cells with the recombinant retroviruses was achieved. The results of rickettsial challenges of adaptein-producing RBE4 cells are very encouraging. Successful transfection of RBE4 cells with the pNF-kB-d2EGFP plasmid and demonstration of NF-kB activation after rickettsial infecton were also achieved. Great progress was made in development of in vitro models of endothelial barrier using rat-derived and human derived brain microvascular endothelail cells. Demonstration of the role of cytokines in modulation of microvascular permeability and possible role of ROS, calcium and NO in endothelial permeability was also demonstrated.

14. SUBJECT TERMS
Rickettsiae, orientia, adapteins, scrub, and epidemic typhus

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I.   INTRODUCTION

Rickettsiae are obligately intracellular organisms that have evolved in close association with an arthropod host. Diseases caused by these organisms are still prevalent in many parts of the world and include Rocky Mountain spotted fever (the most common rickettsiosis in the US), epidemic and endemic typhus (1-4). The latter two are still responsible for thousands of deaths around the world every year. Rickettsial diseases are found in every continent except for Antarctica and are considered both emerging and re-emerging infectious diseases. In addition, *Rickettsia prowazekii* and *R. rickettsii* are listed in the Select Agents Act and are part of the Centers for Disease Control and Prevention (CDC) and NIH category B agents and the North Atlantic Treaty Organization (NATO) select agent list for their potential use as bioterrorist/biowarfare agents (5-8). The most feared complications of rickettsial infections are the development of severe cerebral and pulmonary edema leading to permanent neurologic sequelae or death owing to respiratory failure (3,4). The target cell of rickettsial pathogens is the endothelium lining the vessels of the microvasculature, as demonstrated by studies performed on autopsy cases dying of rickettsioses and animal models (9,10). The purpose of this study is to utilize adaptein libraries coded within pantropic retroviral vectors that confer protection against rickettsial pathogens. In addition, molecular pathogenesis of rickettsioses is being studied by developing *in vitro* models to study endothelial permeability and intracellular rickettsial killing in both wild type and adaptein protected cells. The long term objective of this proposal is to develop new treatments for rickettsioses and to identify novel molecular targets of rickettsial pathogenesis that would provide sites for new therapeutic interventions, and to eventually use these targets to develop effective and rapid BWT countermeasures. The new therapeutic interventions are justified due to the narrow range of antimicrobial agents available for rickettsiae and *Orientia,* emergence of chloramphenicol- and tetracycline-resistant strains of *Orientia,* and the possibility of genetically engineered resistance.

II.   RESEARCH PROGRESS

During the second year of funding for this project, we have been able to continue the experiments related to specific aims 1 and 3 and have started the experiments on specific aim 2.

A. Specific aim # 1: To utilize retrovirally encoded adapteins to generate cell monolayers resistant to rickettsial challenge.

<u>Generation of combinatorial adaptein libraries</u>: Specific details about the construction of the libraries can be found in our first progress report. During the second year, large-scale production of the libraries continued in order to perform the challenge experiments.

In our previous report we discussed methods to amplify small numbers of productive retroviral inserts, to propagate these and then to isolate the useful clones encoding pathogen resistance factors. This is shown in Figure 1. It is based on established methods used for amplification of useful clones from phage display libraries and involves infecting cells with a retroviral vector and then recovering progeny by regenerating the retroviruses by transfection with rescue plasmids.

The method works well, and we were able to amplify small numbers of retroviral inserts this way (Figure 2). Cells infected with a β-galactosidase encoding retrovirus at very low MOI of 0.01 incorporated this gene at 1/100 cells. A single round of amplification recovered all input virus. Each successive round gave a 10-fold amplification. We have applied this technique in our assays, and it shows some promise, but we feel it may not be necessary now as we are obtaining success with our viral screens using our initial standard method and the new library construct below. This was achieved essentially by optimizing our assaying and recovery techniques.

<u>EGFP scaffold protein.</u>

Peptides linked to enhanced green fluorescent protein (EGFP) have been shown to alter cellular signaling mechanisms (11, 12). This scaffold has other advantages over our original designs as well. The fluorescent scaffold is

visible by microscopy and is also highly soluble. We therefore constructed combinatorial libraries by linking an EGFP scaffold to a C-terminal randomized peptide adduct of 6, 12, or 18 amino acids. Retroviruses have now been produced, and titers and library complexities exceed 10^8. This library was successfully tested by application to isolating Sindbis virus resistant cells. Five virus-resistant clones have been characterized now and their altered gene transcription pathways mapped by gene-chip analysis (Davey R., *et.al.*, submitted for publication, PNAS). This test system has allowed us to troubleshoot and develop a robust set of methodology and analytical expertise that is now being applied to *Rickettsia*. We have been using this second library for all experiments since it allows us to track expression of the library directly.

Rickettsial challenges:

All challenge experiments have been performed with combinatorial 6-, 12-, and 18-mers (adapteins), and the diversity has ranged between 2 x10^7 to 3-5 x 10^8. During the first year of the project, we used SVEC cells and Vero cells for all challenges, and each of these cell types had its drawbacks and made interpretation of the challenge experiments difficult (see previous progress report). For the second year, and after optimization of challenge protocols, we have decided to use a rat-derived brain microvascular endothelial cell line (RBE4) developed by Dr. Francoise Roux in France and used for the first time last year as part of our experiments in specific aim #3. The rationale for this change was the lower mitotic capacity when compared to Vero cells and SVEC cells and the presence of "looser" junctions between cells, as opposed to Vero cells, so that selection of the resistant phenotypes was more evident and efficient. In addition, with this cell line, use of sulfoximine or any other chemicals to enhance cell mortality was not necessary.

Cells were infected at a MOI of 25 with renografin-purified *R. rickettsii*. At 96 hours after the challenge, surviving cells still attached to the substratum were trypsinized, re-plated, and allowed to grow for 48 to72 hours before being re-challenged with *R. rickettsii* (MOI = 15). Three more rounds of re-

challenges (MOI = 10) were carried out. At the end of 5 rounds of challenges, several foci of putative rickettsia-resistant RBE4 cells were recovered. However, none of the surviving cells in these initial experiments were EGFP-positive suggesting that the cells had lost expression of the adapteins encoded in the retroviral vector. We then performed PCR to try to amplify a target sequence within the retroviral vector, and no amplification products were obtained suggesting that the "survivors" had not been infected with the retroviral library from the beginning of the experiment. The cells were frozen in glycerol and kept at -80 °C to characterize them at a later date and elucidate their resistant phenotype. However, when the cells were thawed and plated, no cell growth occurred. The experiment was repeated several times exactly as described and after the first challenge, a number of EGFP-positive cells (10 cells approximately), together with some EGFP-negative cells, were recovered (Figure 3). These cells were wrought to confluency in a T-25 flask (Figure 4) and re-challenged with R. rickettsii for the second time. Second-round survivors were trypsinized and re-plated in two wells of a 12-well plate. A typical "colony" comprising mostly, if not all, EGFP-positive cells (approximately 20-25 cells) grown in one of the wells is shown in Figure 5. Characterization of these EGFP-positive, rickettsia-resistant cells is currently underway. However, one of the obstacles we are facing with these cells is their lower mitotic capacity that makes expansion of these cell "clones" difficult. Enrichment of the culture medium will be attempted in order to increase the mitotic capacity of these resistant cell "clones". Initial challenges with R. prowazekii and O. tsutsugamushi are yielding similar results. We are currently continuing with further challenges. Two additional immortalized human brain microvascular endothelial cell lines (SV40 and telomerase transformed) have been acquired by our laboratory and will be evaluated in rickettsia-challenge experiments. The results with these human cells would be more relevant to human rickettsioses.

B. Specific aim #2: To determine the roles of NF-κB, cytokines (TNF-α, IFN-γ, RANTES), ROS and NO in intracellular killing of rickettsia-resistant monolayers.

A total of 10^5 cells were seeded in 24-well plates and transfected at semiconfluency with pNF-κB-d2EGFP vector plasmid (Clontech) by using LipofectAMINE PLUS reagent. Optimization of the transfection protocol was performed according to manufacturer's instructions by using different concentrations of plasmid DNA (range:1.0-1.6 μg). The transiently transfected cells were infected with *R. rickettsii* at 10 MOI and were then monitored under an inverted Olympus microscope equipped with UV light and EGFP filters. Cells were evaluated every 30 minutes for 2 hours. NF-κB activation was demonstrated in infected cells as emission of green fluorescence (Figure 6A-B). Negative controls included in the experiment included non-transfected and non-infected RBE4 cells. Experiments with rickettsia-resistant monolayers will be started as soon as we manage to expand our rickettsia-resistant "clones" more efficiently.

C. Specific aim #3: To characterize signal transduction pathways modulating the cytoskeletal events responsible for the increased vascular permeability seen in rickettsial infections.

RBE4 cells as well as human-derived primary cerebral endothelial cells (HBMEC, Applied Cell Biology Research Institute, Kirkland, WA) were seeded on disposable arrays with gold electrodes at a density of 10^5 cells. Resistance across the cell monolayer (transendothelial resistance or TER) was monitored with Electronic Cell-substrate Impendance Sensing (ECIS, Applied Biophysics, NY) until TER stabilization occurred. The monolayers were then infected with 10 MOI of *Rickettsia rickettsii*. After rickettsial internalization, cytokines (IL-1-β, TNF-α, and IFN-γ), either singly or in combination, were added at low (5 ηg/ml) and high (20 ηg/ml) concentrations to the infected and non-infected monolayers. Controls in these experiments included gold electrodes containing growth medium alone and non-infected cell monolayers.

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All experiments have been performed in triplicate. The results were similar for both RBE4 cells and HBMEC cells and can be summarized as follows:

TER across the cell monolayers was higher in HBMEC monolayers as compared to RBE4 cells due to expression of tight junction phenotype as evidenced by demonstration of ZO-1 by immunofluorescence (Figure 7). Increase in vascular permeability as demonstrated by a decrease in TER was observed after 60 – 70 hours (RBE4) or 75 – 80 hours (HBMEC) of rickettsial infection at a MOI of 25 (Figures 8 and 9). At these time points cell micromotion was still evident, suggesting that cell viability was not greatly compromised at least during the initial phases of decreased TER across the cell monolayer. By days 6 and 7 PI, the infected cell monolayer lost its viability as evidenced by loss of micromotion in the electrical traces. In addition, the decrease in TER was directly proportional to the rickettsial inoculum (Figure 10). Drop in resistance was noted at 65 hours post-infection in all monolayers. However the change in resistance was more drastic as evidenced by the steeper slopes in cell monolayers infected with higher MOI.

The presence of cytokines potentiated an earlier and more dramatic decrease in TER than the monolayers infected with *R. rickettsii* alone. Low doses of TNF-α decreased resistance significantly when compared to infected cell monolayers without the cytokine. High doses of TNF-α also decreased resistance significantly but appeared to induce cell death (loss of micromotion) earlier (Figures 11 and 12). IL-1β decreased resistance earlier and faster than rickettsiae alone. Decreases in resistance were similar to the ones caused by low levels of TNF-α (Figures 13 and 14) Both high and low levels of INF-γ induced lower resistance across the monolayers (more pronounced at low levels). Cell survival (preservation of micromotion) was prolonged by the cytokine, especially at low levels (Figures 15 and 16). Addition of IL-1β and TNF-α revealed lower maximum resistance levels across the cell monolayers than values obtained with rickettsia-infected and cytokine-free monolayers. The rate of resistance decrease was gradual and cell survival in the monolayers (preservation of cell micromotion) was prolonged

(Figures 17 and 18). Addition of TNF-α and IFN-γ yielded similar results (Figures 19 and 20). An early drop in resistance was noted after addition of low levels of both IFN-γ and IL-1β. The overall resistance throughout the experiment was lower than monolayers without this pair of cytokines. Decrease in resistance was gradual and cell viability increased (Figures 21 and 22). Addition of all three cytokines induced marked decrease in resistance throughout the experiment and cell viability was lost early in the time course (Figures 23 and 24).

In order to further elucidate the possible effects of ionized calcium, ROS and NO on endothelial permeability several chemicals were added to infected and non-infected cell monolayers. These chemicals included ROS scavengers such as catalase (10 U/ml) and tocopherol (30 µM); a glutathione replenisher (N-acetyl cysteine or NAC, 1 mM); an inhibitor for the inducible form of nitric oxide synthase or iNOS (aminoguanidine or AG, 200 µM); and a cytoplasmic calcium chelator or BAPTA-AM (20 µM). Experiments were performed using RBE4 cells and another rat brain derived microvascular endothelial cell line (GP8). Only one set of experiments has been performed, and the results are preliminary. Tocopherol, AG, and BAPTA-AM did offer protection against increased endothelial permeability as demonstrated by a higher TER in monolayers pretreated with such chemicals (Figures 24 and 25). Experiments with cytokines and these chemicals are also planned in the very near future.

As mentioned before, we recently acquired two previously unavailable immortalized brain microvascular endothelial cell lines of human origin. Preliminary data indicate that these endothelial cell lines maintain their characteristics of forming tight junctions *in vitro* (immunohistochemical demonstration of tight junction proteins and a high TER in the range of 1500 Ohm). ECIS experiments will also be performed with these cells.

III. KEY RESEARCH ACOMPLISHMENTS:

Construction of a second set of libraries containing 6-, 12-, and 18-mer peptides and EGFP in the retoroviral vector.

Successful transfection of rat derived brain microvascular endothelial cells and human brain primary microvascular endothelial cells with the recombinant retroviruses.

Encouraging results with rickettsial challenges of adaptein-producing RBE4 cells.

Successful transfection of RBE4 cells with plasmid and demonstration of NF-κB activation after rickettsial infection.

Great progress in development of *in vitro* models of endothelial barrier using rat-derived and human derived microvascular endothelial cells. Demonstration of the role of cytokines in modulation of microvascular permeability and possible role of ROS, calcium and NO in endothelial permeability.

IV.   REPORTABLE OUTCOMES

1. Koo P, Olano JP. Analysis of microvascular leakage in an *in vitro* model of the brain endothelial barrier by electric cell-substrate impedance sensing (ECIS). American Society for Rickettsiology. Abstract Book. 2003. To appear in the Department of Defense Peer Reviewed Medical Research Program Investigators Meeting. Abstract Book, 2004.

2. Koo P, Watowich S, Davey R, Olano JP. Development of combinatorial peptide based libraries (adapteins) with rickettsicidal activity: preliminary results. American Society for Rickettsiology. Abstract Book. 2003. To appear in the Department of Defense Peer Reviewed Medical Research Program

Investigators Meeting. Abstract Book 2004.

3. Koo P, Wen G, Olano JP. ECIS: A novel approach to measure endothelial permeability in *Rickettsia*-infected cells. Gulf Coast Tropical Medicine Association Meeeting. Texas 2003.

4. Koo P, Olano JP. Characterization of endothelial permeability in rickettsial infections by Electronic Cell-Substrate Impedance Sensing (ECIS). Manuscript in preparation.

V. CONCLUSIONS

In summary, several goals were accomplished during the second year of this project. Challenge experiments have continued and were improved dramatically with the use of a rat-derived brain microvascular endothelial cell line instead of SVEC and Vero cells. The presence of discrete rickettsia-resistant EGFP positive "clones" after several rounds of challenges in RBE4 cell monolayers suggests to us that adaptein producing cells might indeed be resistant to cell death caused by rickettsiae. However, the most immediate challenge we face with these experiments is the efficient expansion of these resistant "cell clones" which to date has not been possible. We are in the process of improving the environmental conditions for cell growth and reproduction. Once we achieve expansion of resistant clones, we will be able to characterize the resistant cell populations. The fact that the adaptein-producing system has been optimized and shown to work with a Sindbis virus model suggests to us that success with rickettsiae is achievable.

Great advances have been made in specific aim #3. ECIS is a powerful technique that has allowed us to evaluate TER on a continuous basis using different human and rat-derived microvascular endothelial cell lines. The results seem to suggest that cytokines play a major role in modulating TER. The mechanisms of action are unknown in our system, but by some publications seem to suggest that they do so by modification of tight and adherens junction

complexes (13-15). Mediators of such changes could include ROS or NO. Indeed, preliminary results with one of our cell lines seem to corroborate the hypothesis that ROS and NO increase vascular permeability as shown by increased TER values when monolayers were pre-treated with tocopherol and amionoguanidine. In addition, intracellular ionized calcium might also play a role as shown by increased TER values in cell monolayers pre-treated with BAPTA-AM.

Activation of NF-κB was also demonstarted in live cells after rickettsial infection. The role of NF-κB in production of ROS and NO is currently being elucidated. Experiments with live cells to study calcium signaling and other cytoskeletal changes with confocal microscopy have unfortunately been delayed this year due to serious technical problems with our current laser confocal microscope. However, our department recently received a generous grant from the Keck Foundation for funding of a state of the art Zeiss LM 510 laser confocal microscope that will be housed in the BSL-3 laboratory. The microscope has already been ordered and we expect to restart the experiments by the summer of this year.

VI.   SCIENTIFIC PERSONNEL

1. David H. Walker, M.D. Principal Investigator (10% effort)
2. Juan P. Olano, M.D. Co-Investigator (25% effort)
3. Stanley J.Watowich, Ph.D. Collaborator (10% effort)
4. Robert A. Davey, Ph.D. Collaborator (10% effort)
5. Paul Koo Ph.D. Post-Doctoral fellow.
6. Olga Kolokoltsova. Research Technician
7. Drew Deniger. Research Technician

No changes in personnel or percent effort from previous year. This second year marks the end of the collaborative effort from Drs. Davey and Watowich. Their role in the project will continue as consultants for the challenge experiments at further improvements of the adaptein technology at no extra cost. Dr. Watowich has requested a no cost extension.

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APPENDICES

Generation of retroviral inserts and adaptein libraries
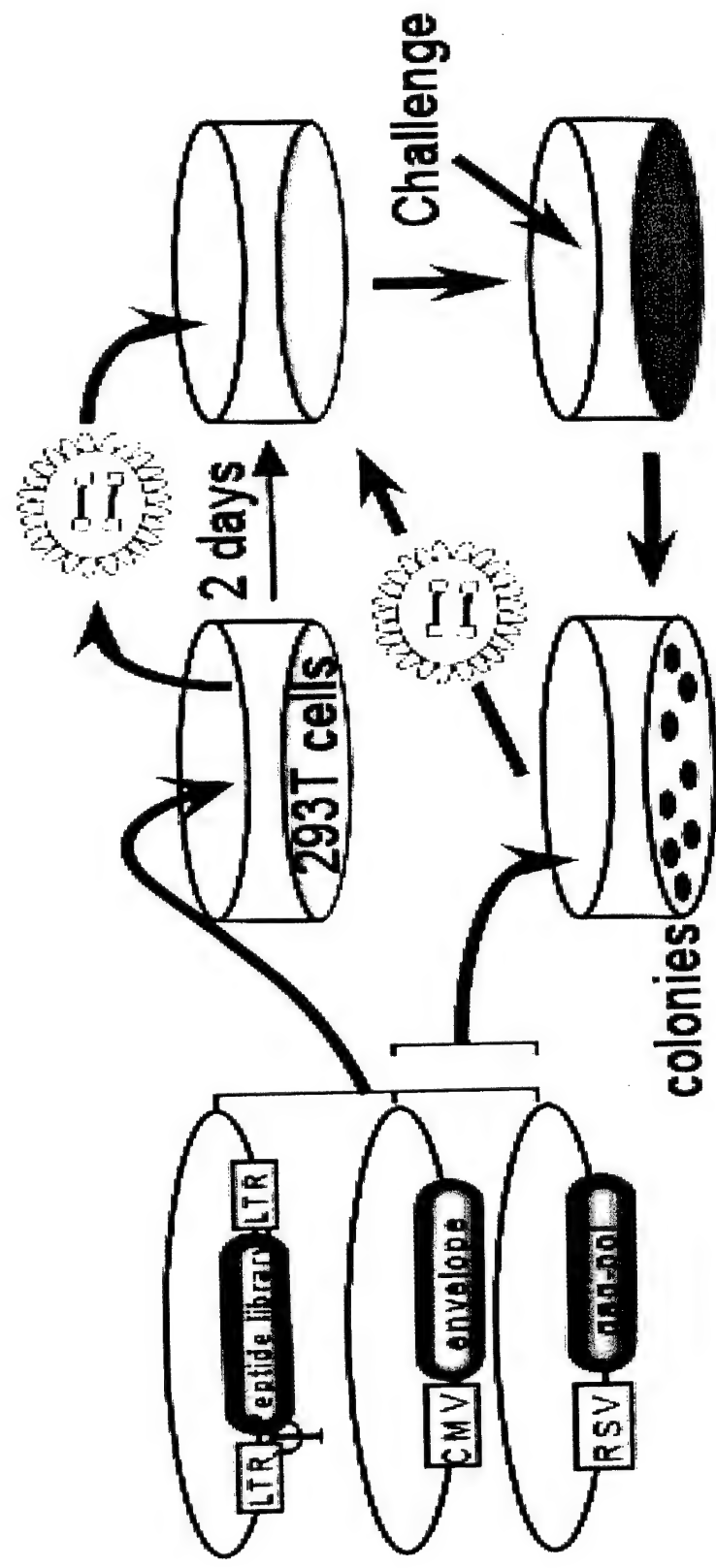
Figure 1

β-galactosidase staining of 293T cells
infected with retroviral inserts

Figure 2

Surviving RBE4 Cells (72 Hours Post-Challenge), 24 Hours Post-Seeding in T-25 (x200)
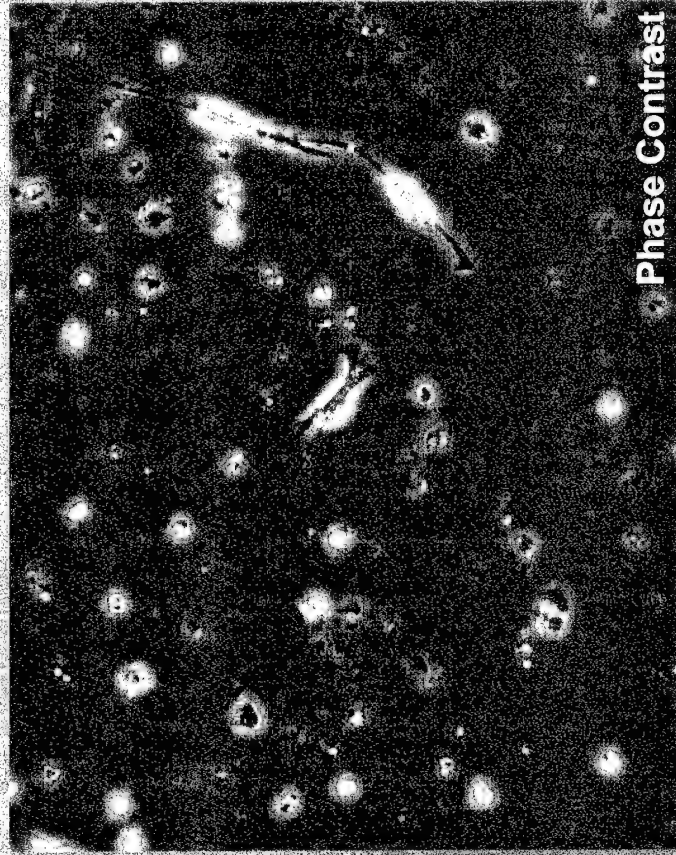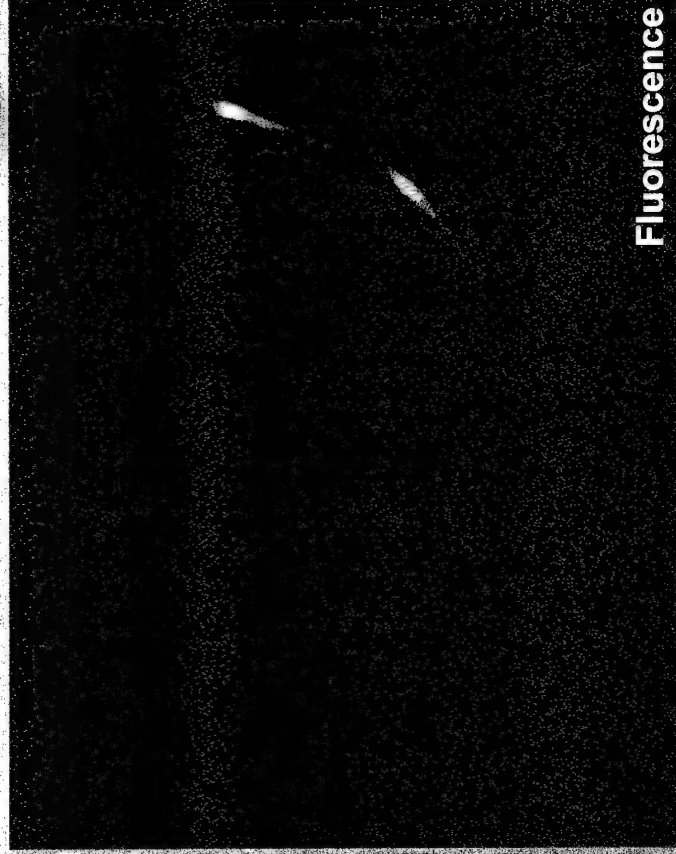
Fluorescence

Phase Contrast

Figure 3

Surviving RBE4 Cells Immediately Before Second Rickettsia Challenge in T-25 (x100)
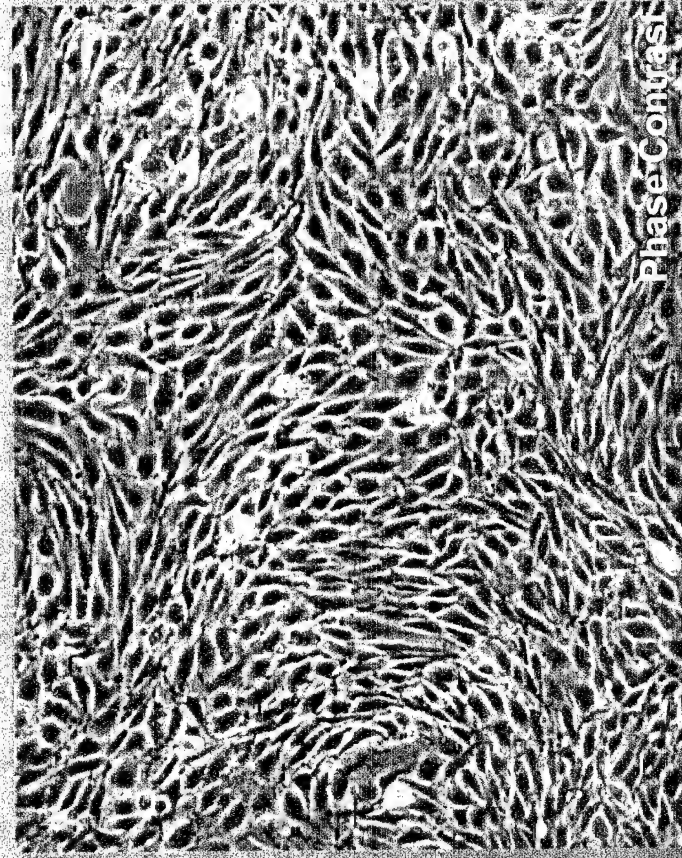
Fluorescence

Phase Contrast
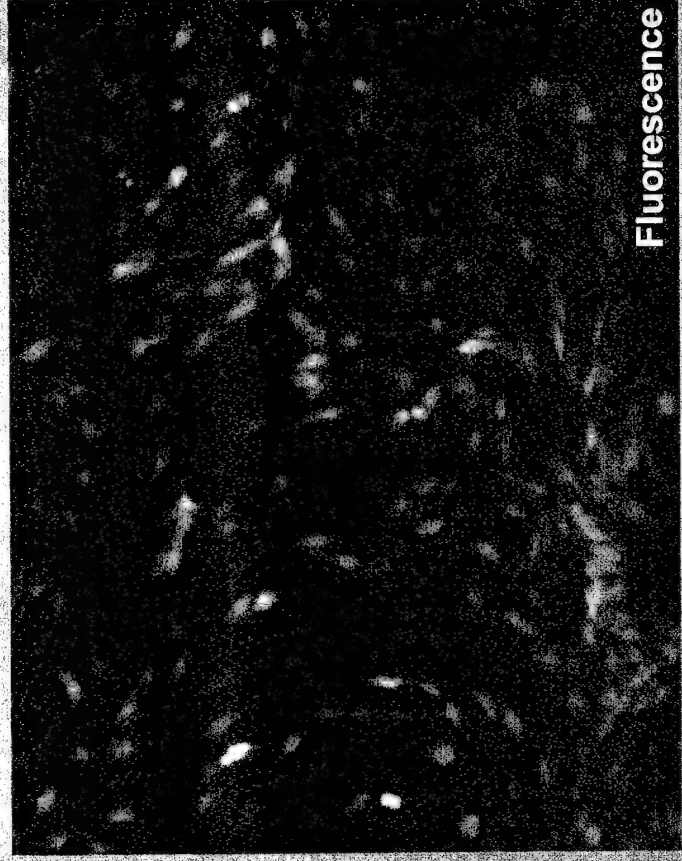
Figure 4

Second Round Surviving RBE4 Cells
24 Hrs After Seeding in 2 wells of a
12-Well Plate (x100)

Fluorescence

Phase Contrast
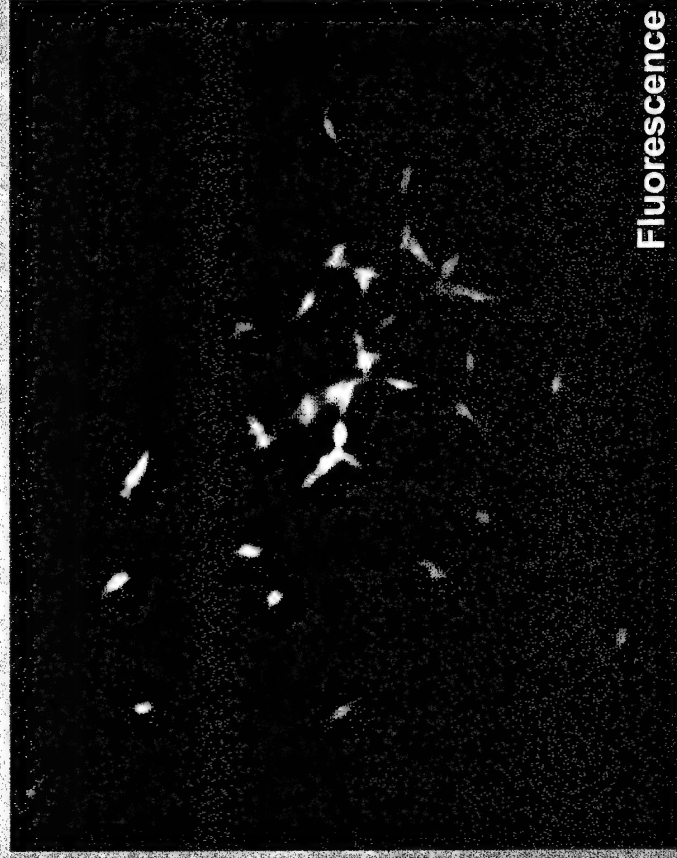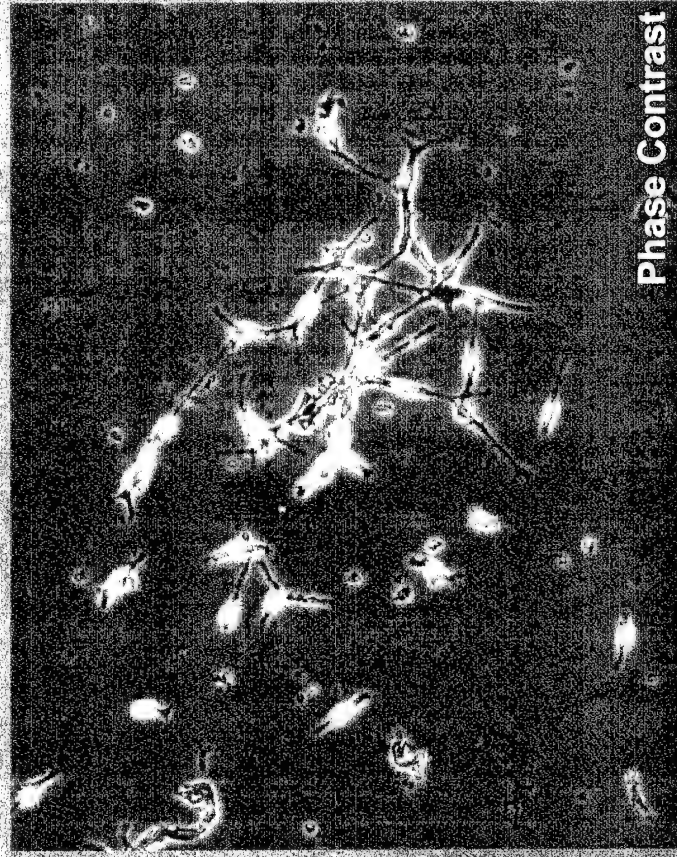
Figure 5

Rickettsia-induced NF-κB activation
Negative control (non-infected and transfected RBE4 cells)

30 min
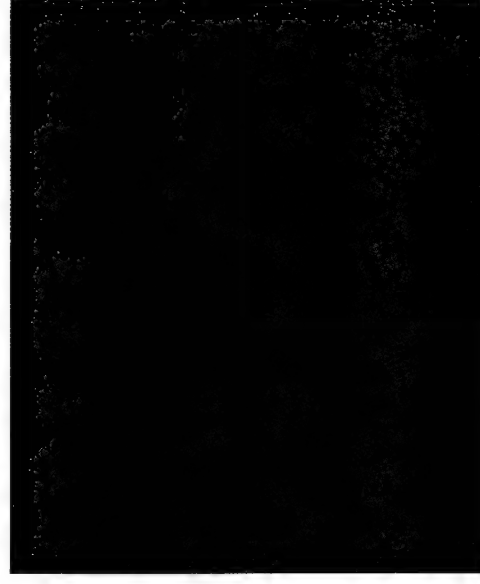
60 min
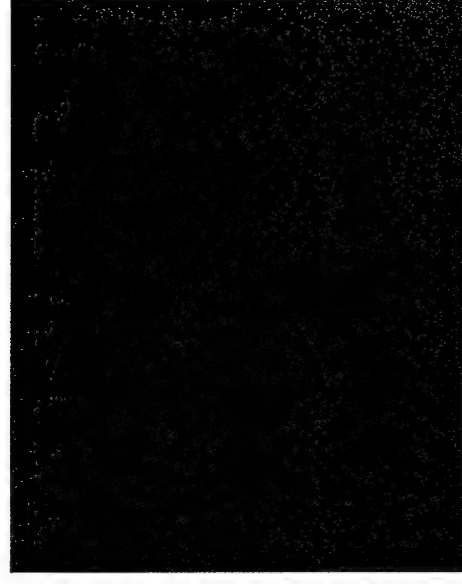
120 min

Figure 6A

Rickettsia-induced NF-κB activation
Transfected RBE4 cells and infected with *R. rickettsii*)

30 min

60 min

120 min
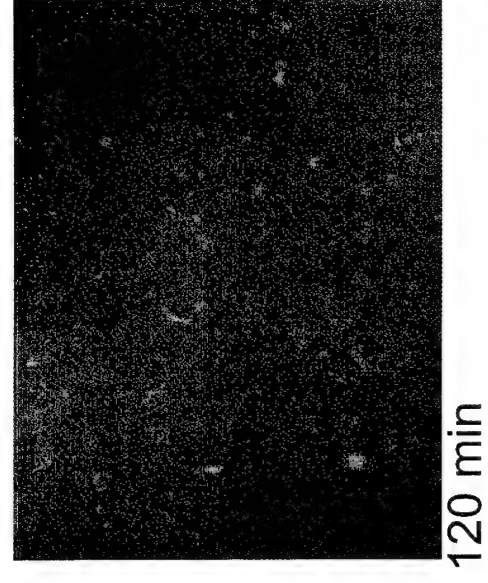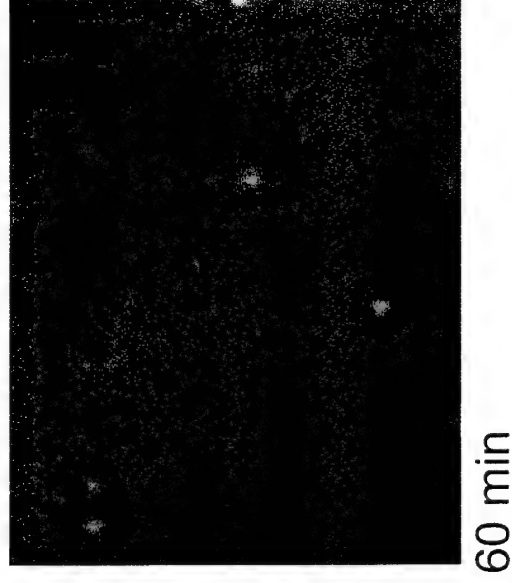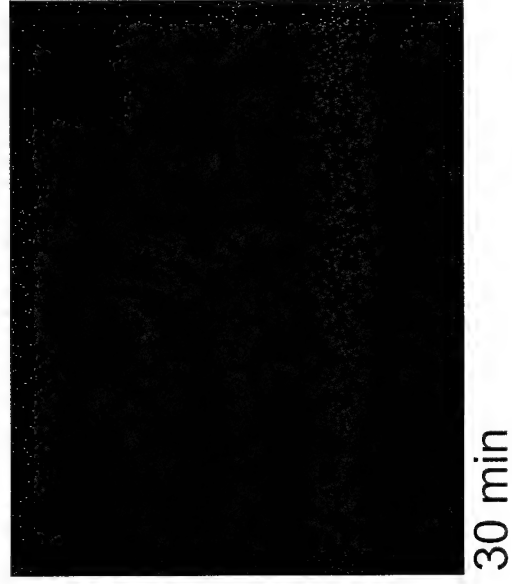
Figure 6B

Demonstration of the Presence of ZO-1 in HBMECs by Immunofluorescence
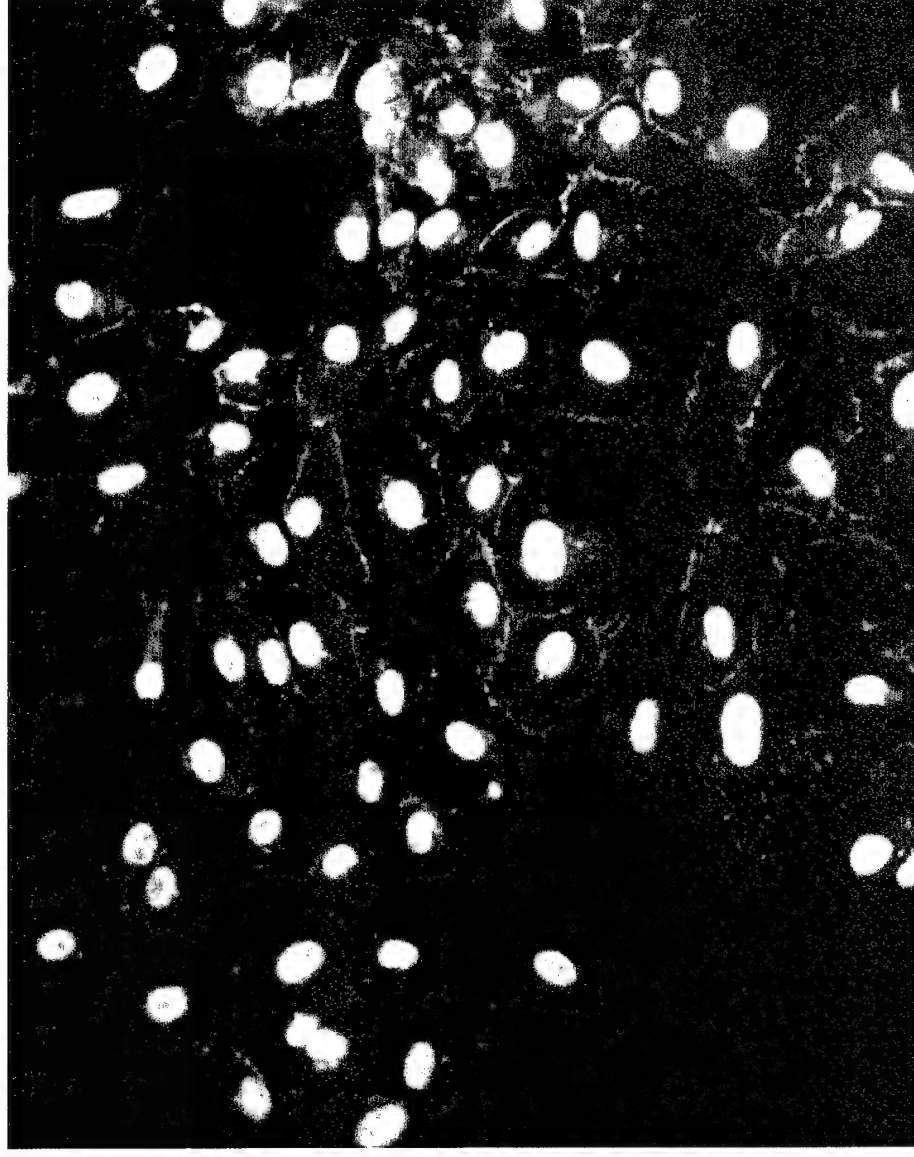
Figure 7

HBMEC Controls (Day 0 – Day 3)
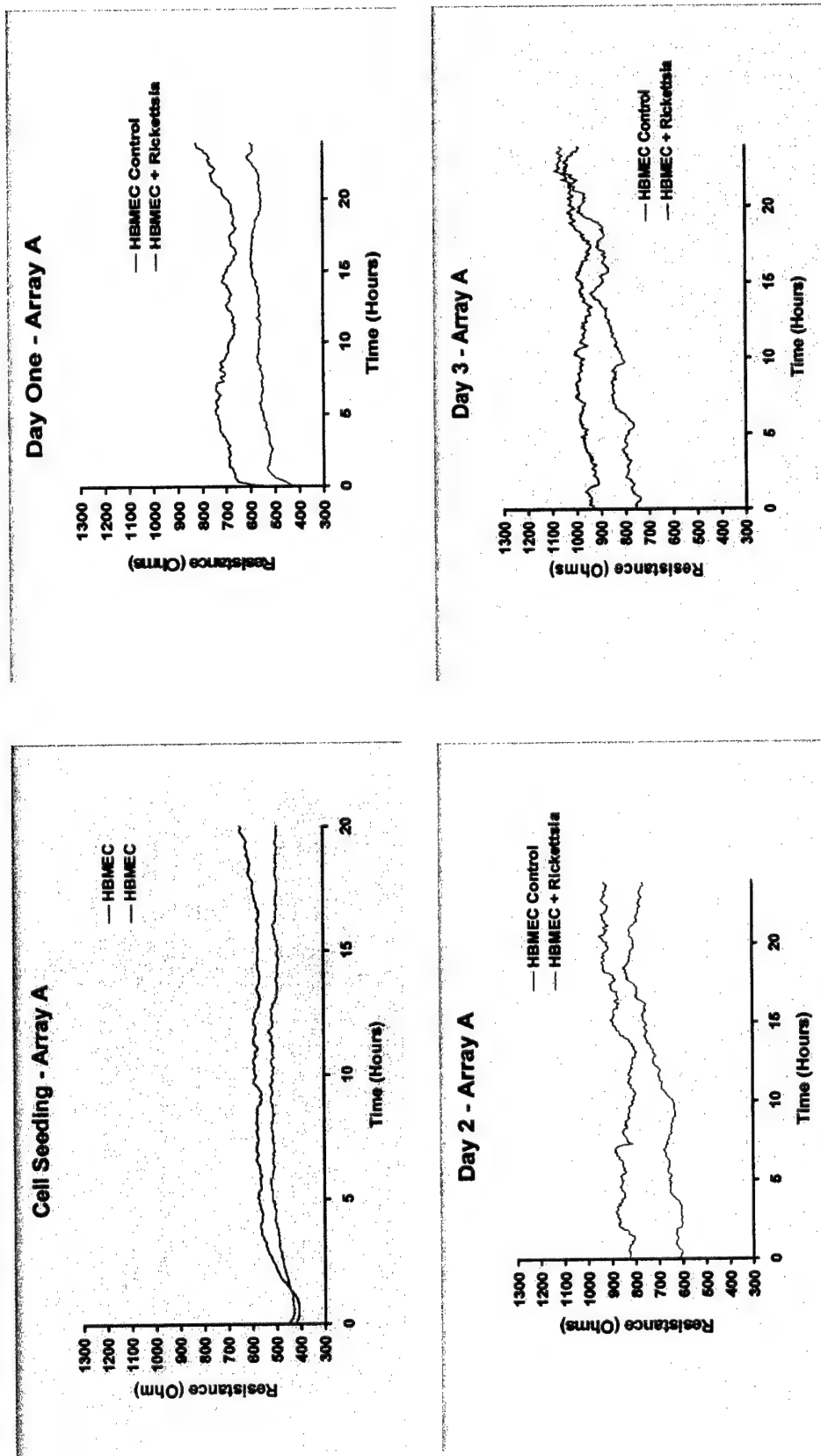
Figure 8

HBMEC Controls (Day 4 – Day 7)

Figure 9

Dose Response of RBE4 Cells to *R. rickettsii* Challenge
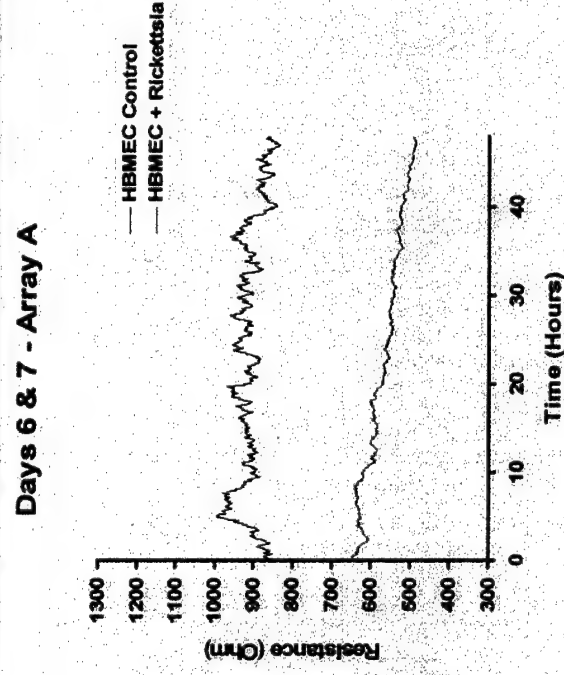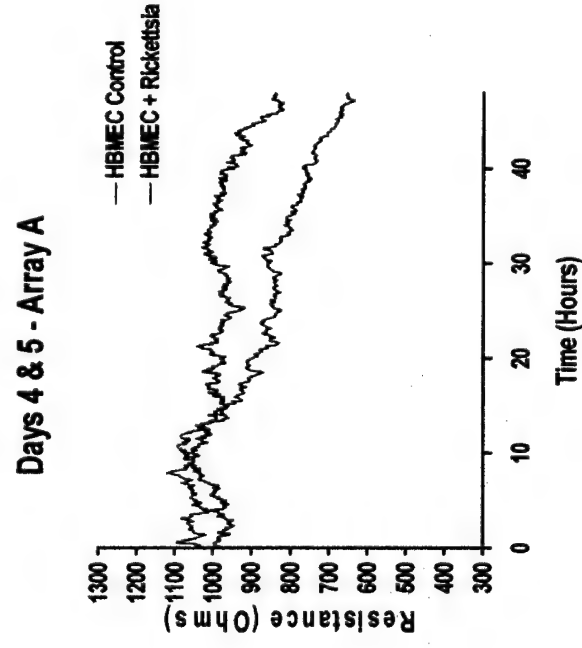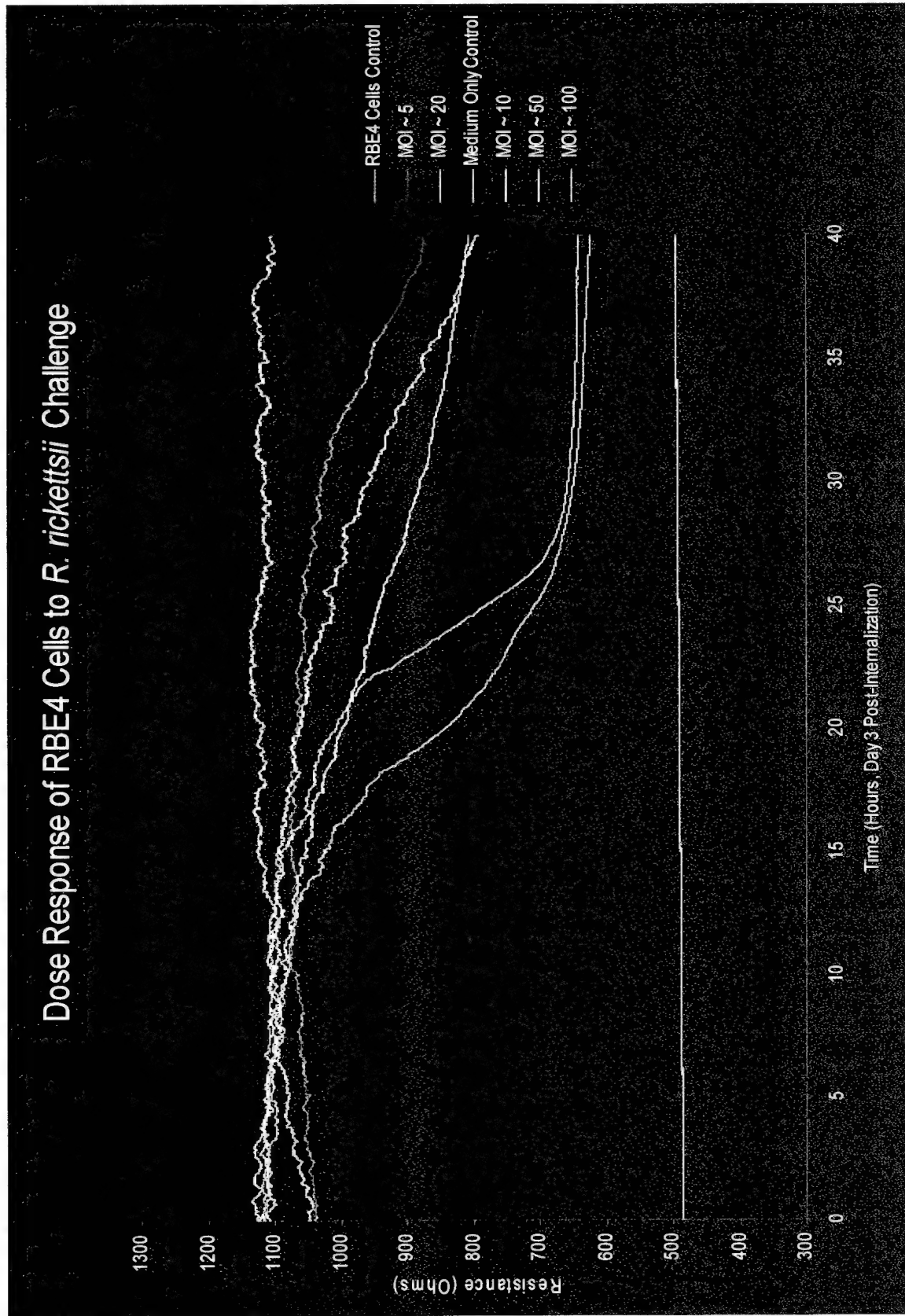
Figure 10

TNF-α (Day 0 – Day 3)
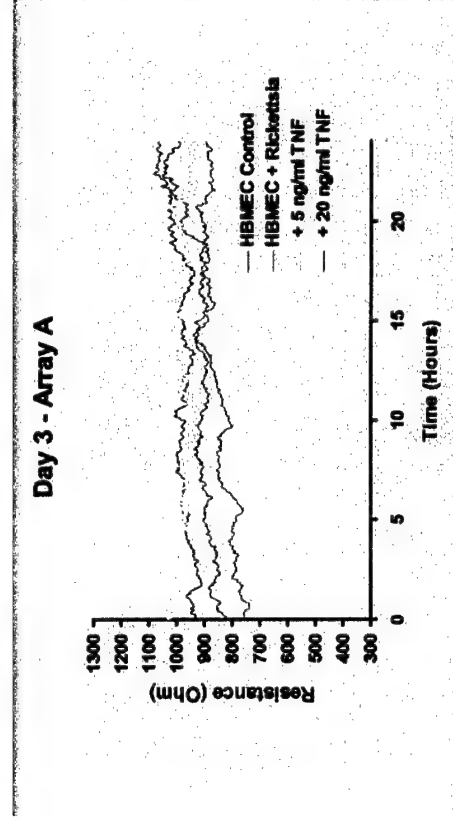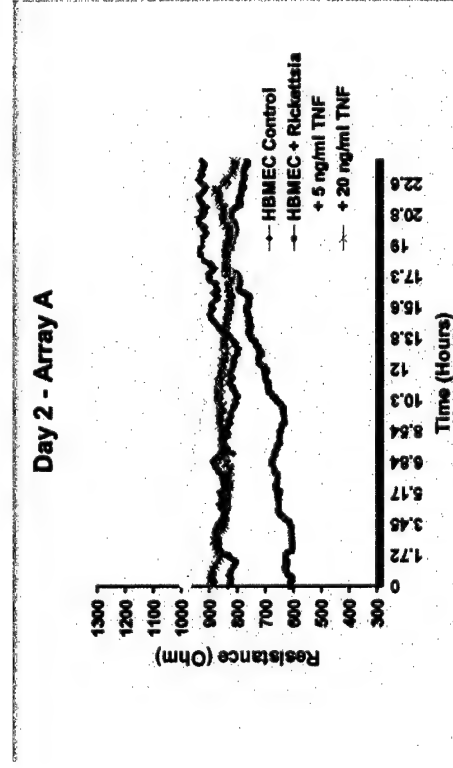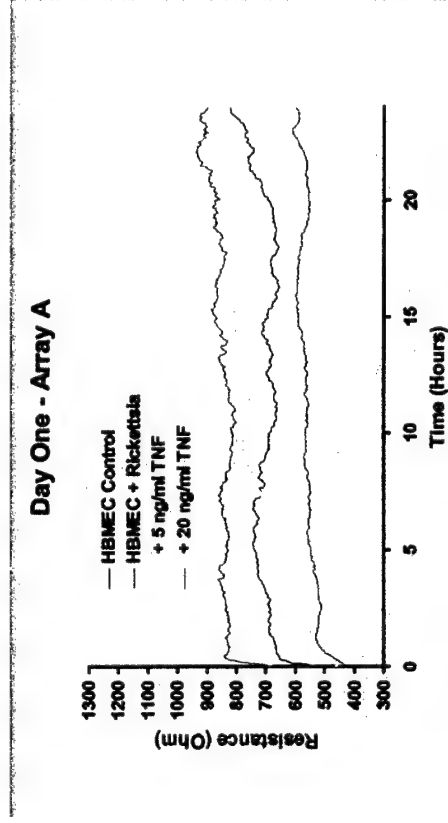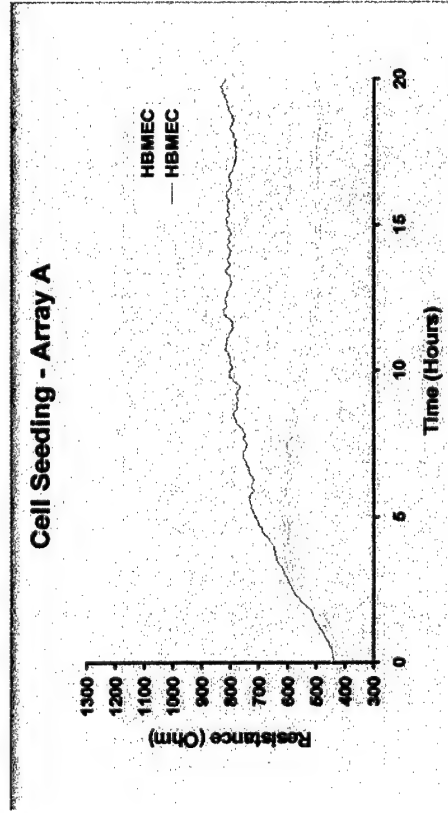
Figure 11

TNF-α (Day 4 – Day 7)
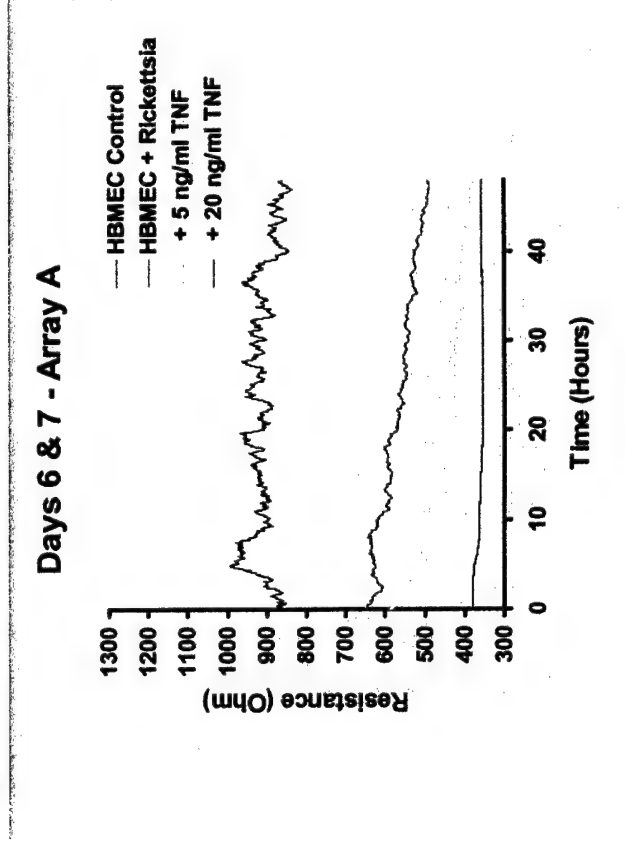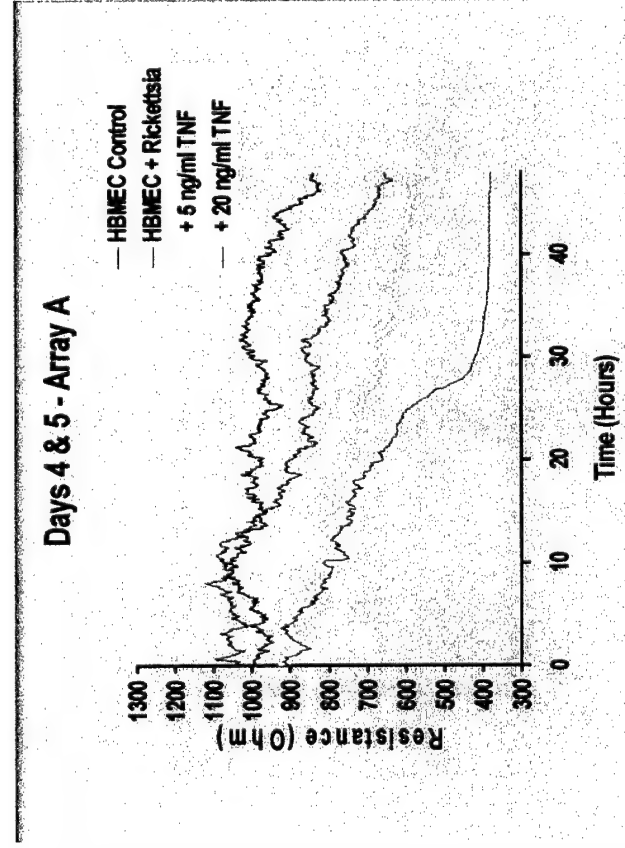
Figure 12

IL-1β (Day 0 – Day 3)
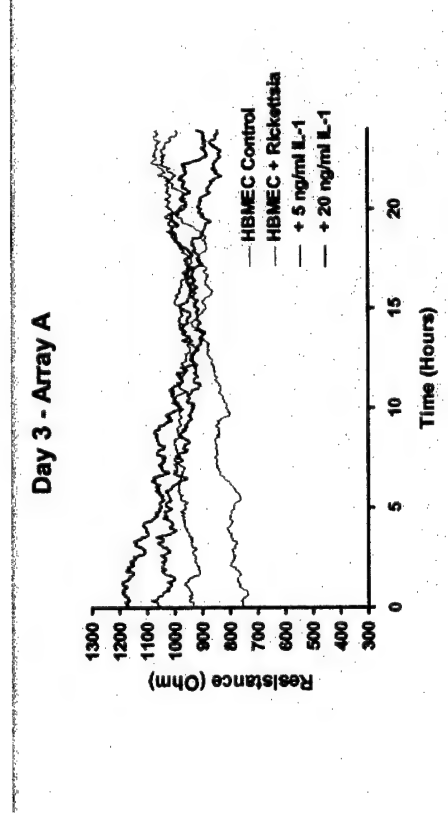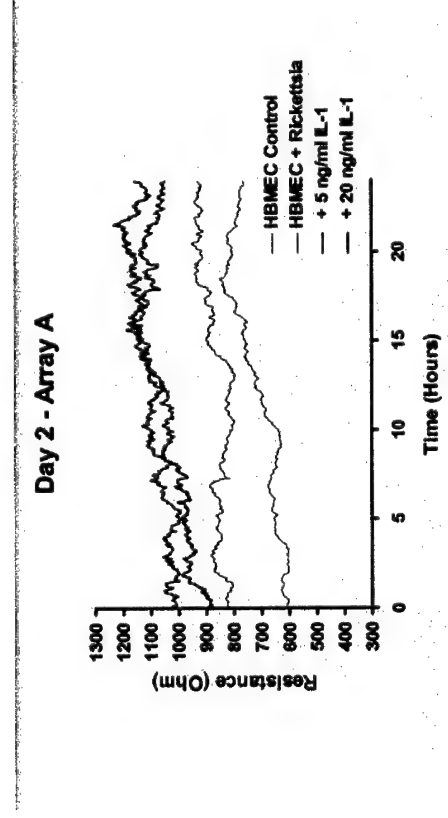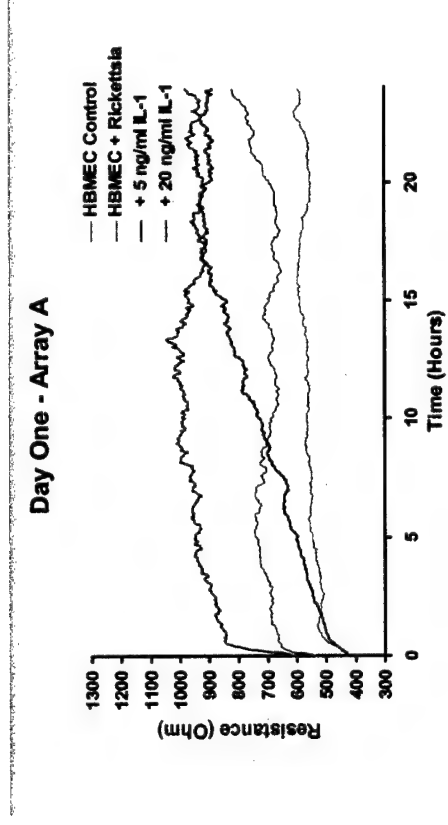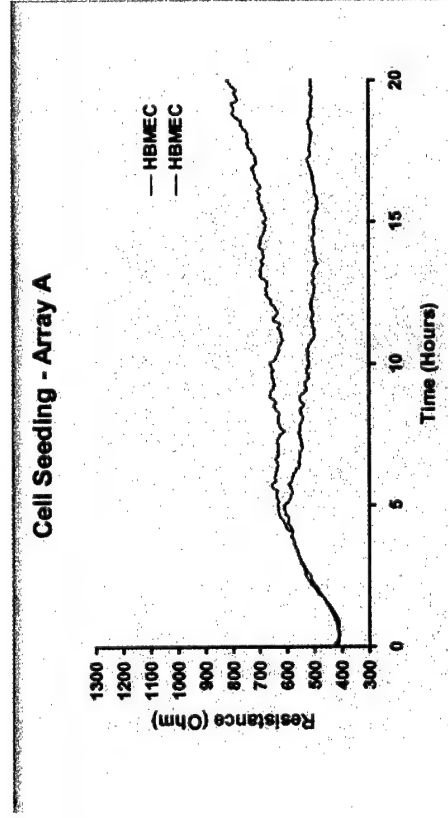
Figure 13

IL-1β (Day 4 – Day 7)
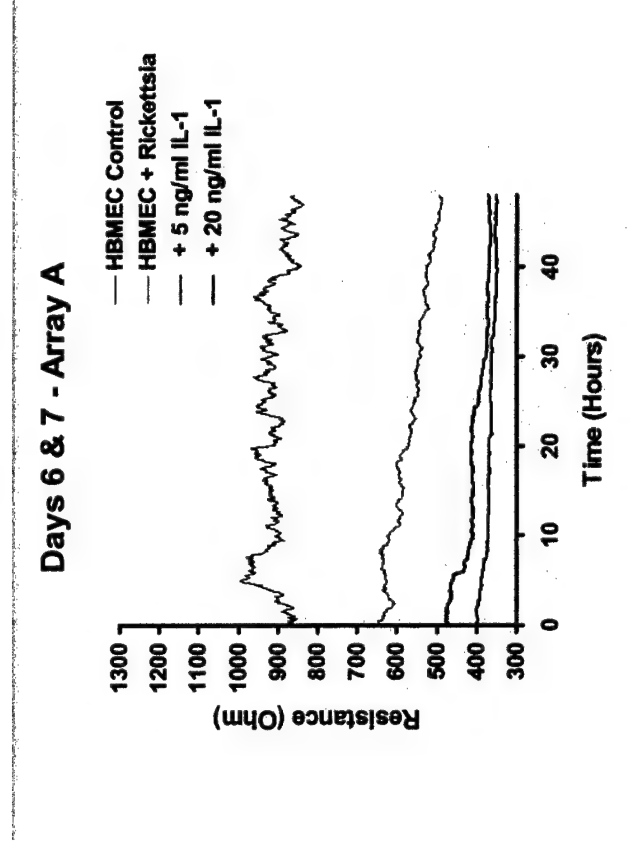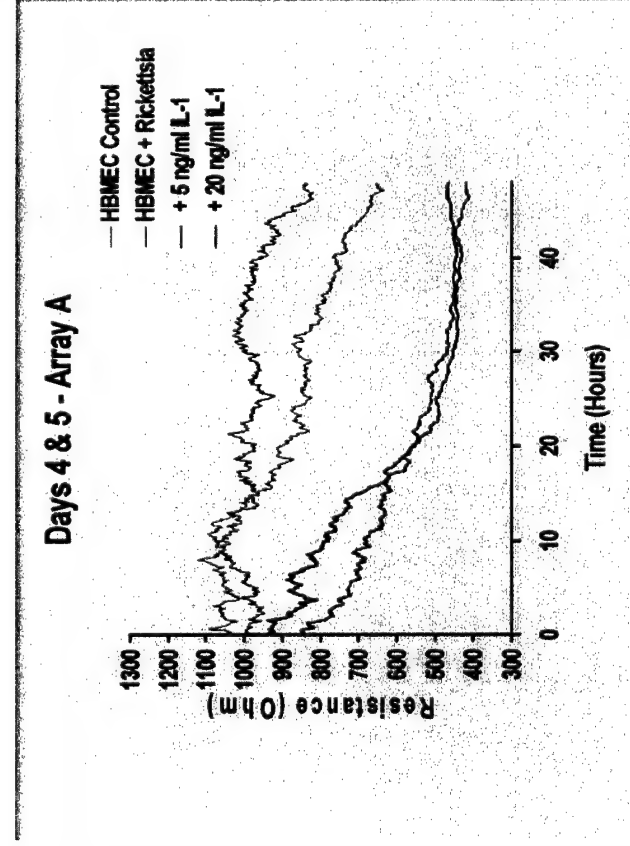
Figure 14

IFN-γ (Day 0 – Day 3)
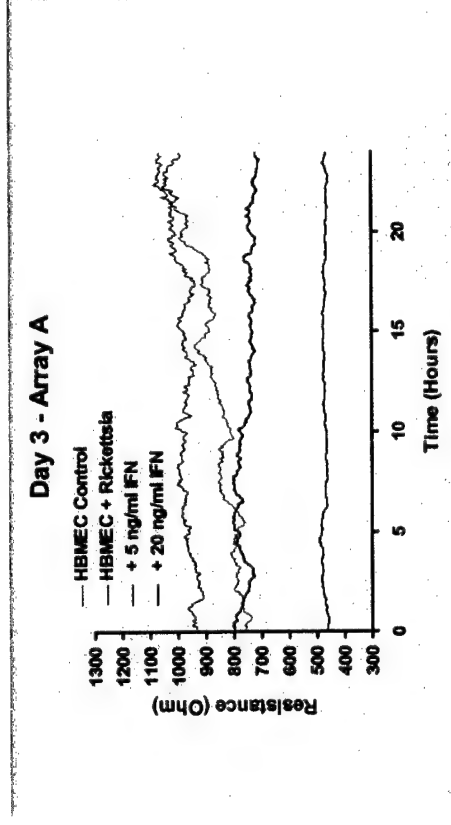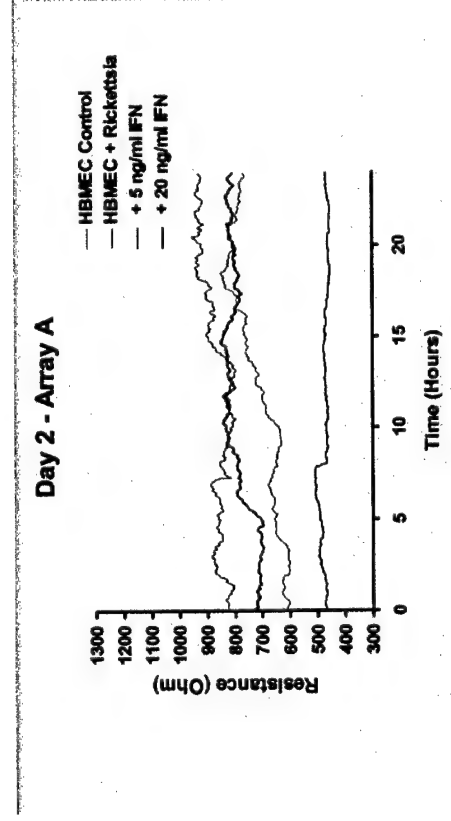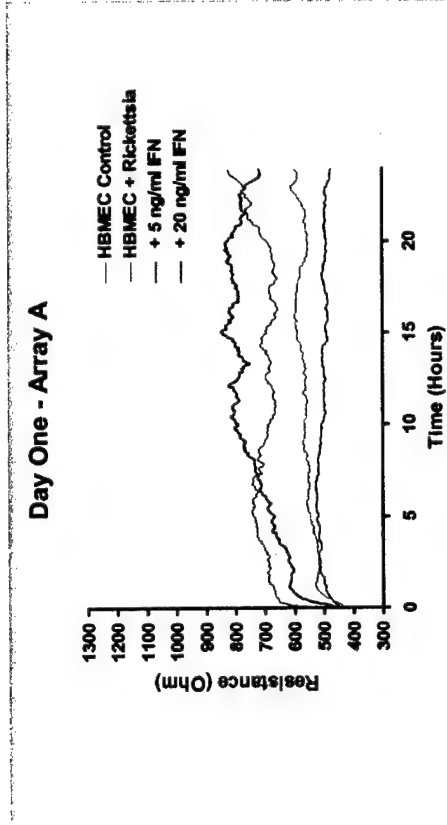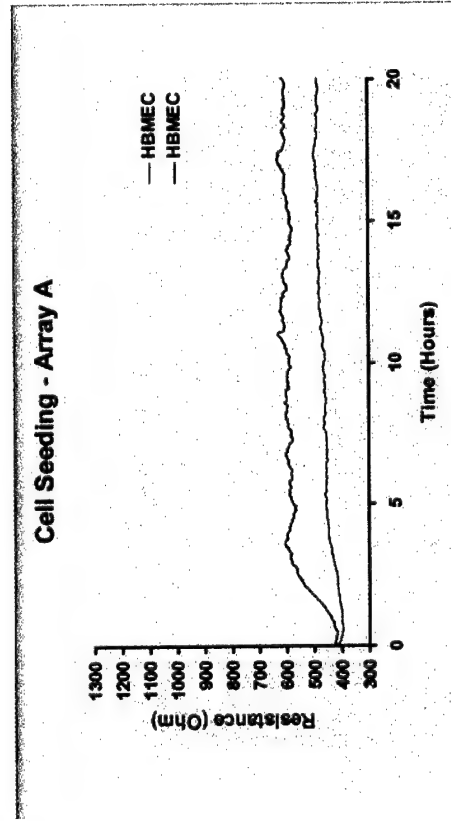
Figure 15

IFN-γ (Day 4 – Day 7)
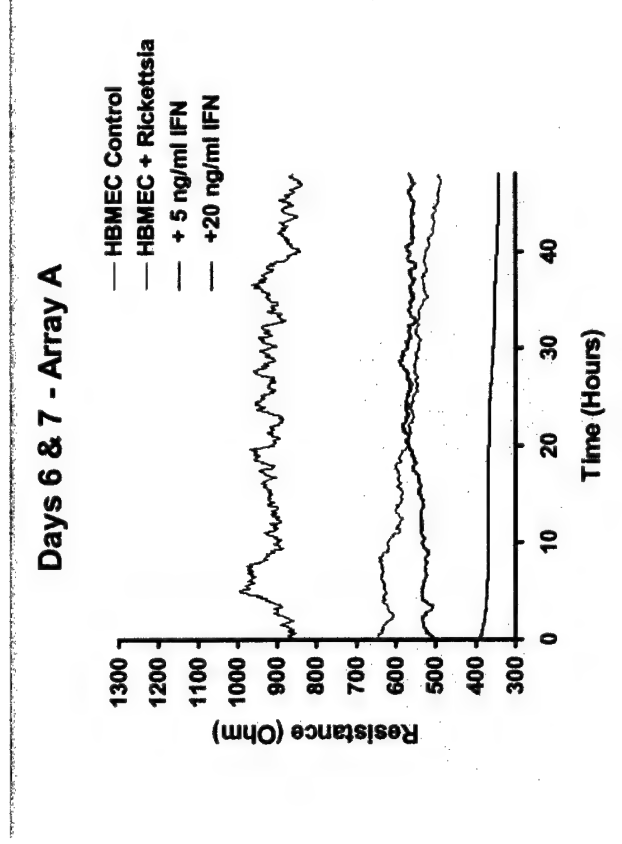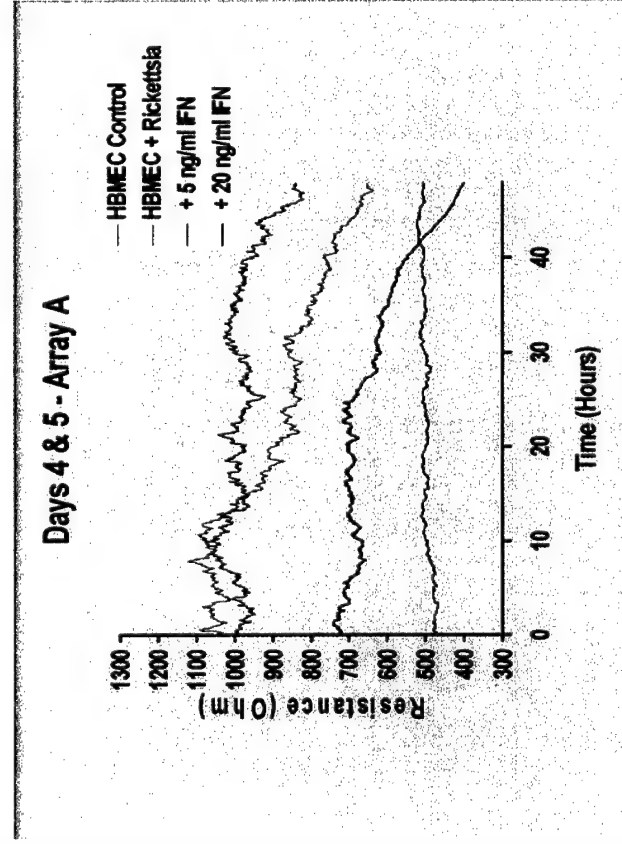
Figure 16

TNF-α + IL-1β (Day 0 – Day 3)
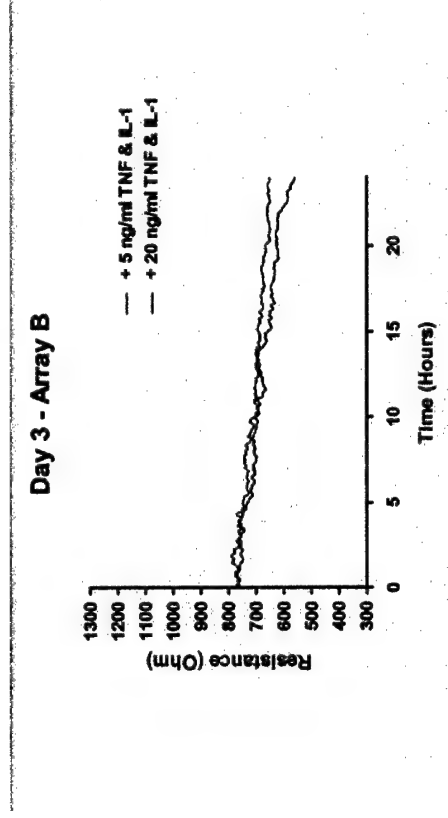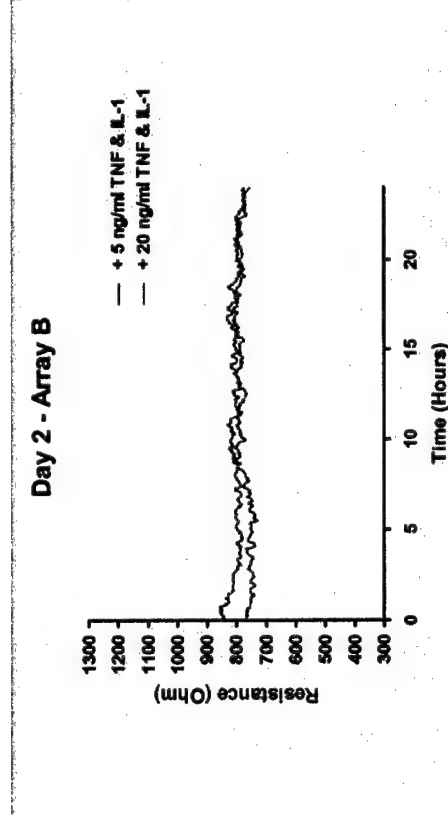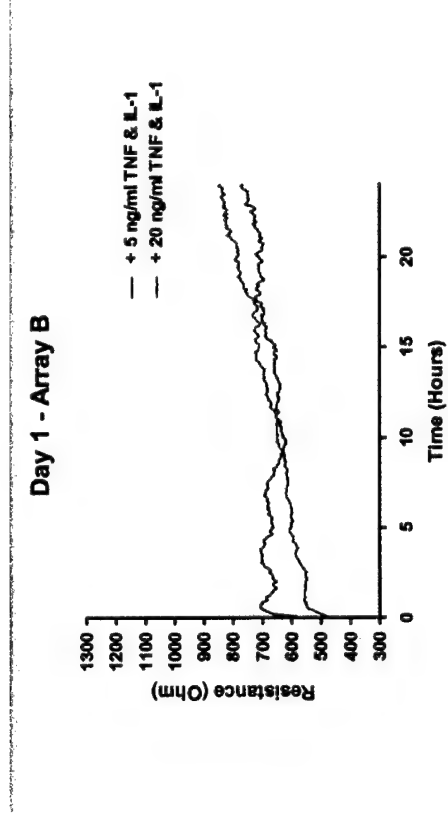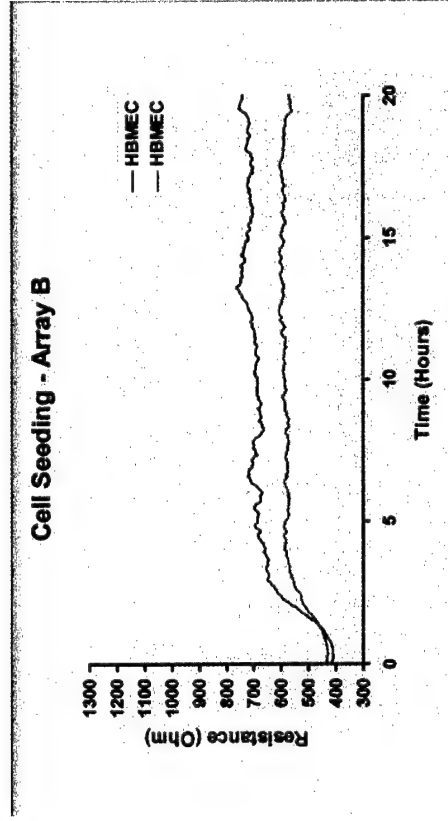
Figure 17

TNF-α + IL-1β (Day 4 – Day 7)
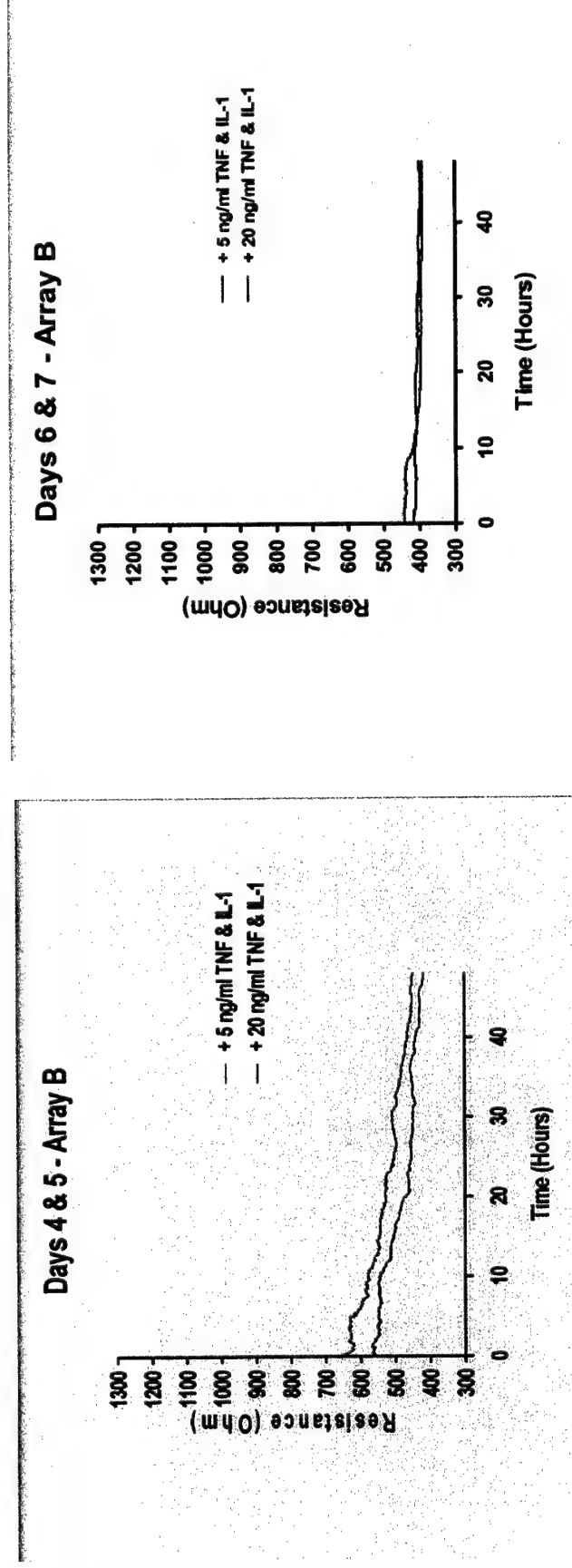
Figure 18

TNF-α + IFN-γ (Day 0 – Day 3)
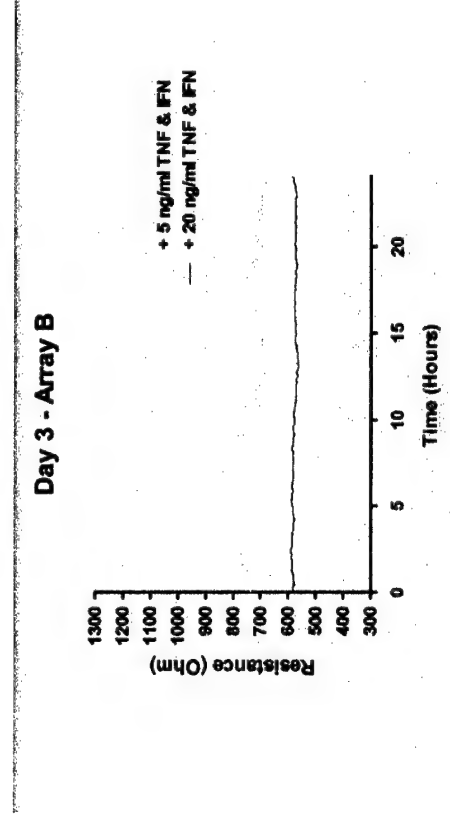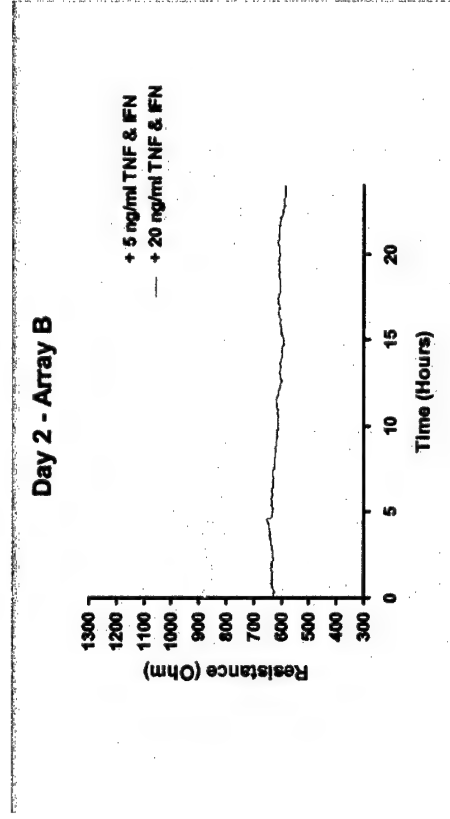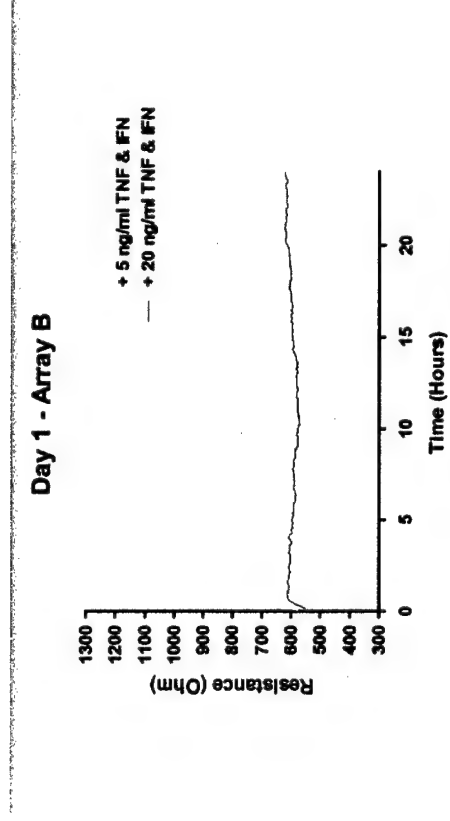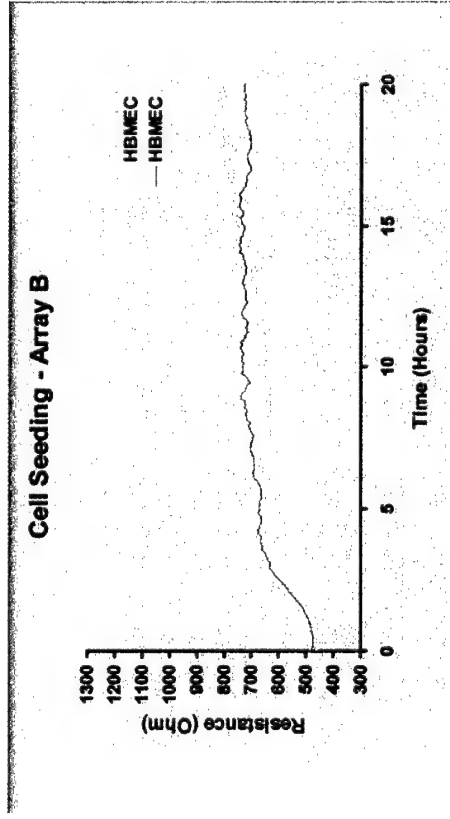
Figure 19

TNF-α + IFN-γ (Day 4 – Day 7)
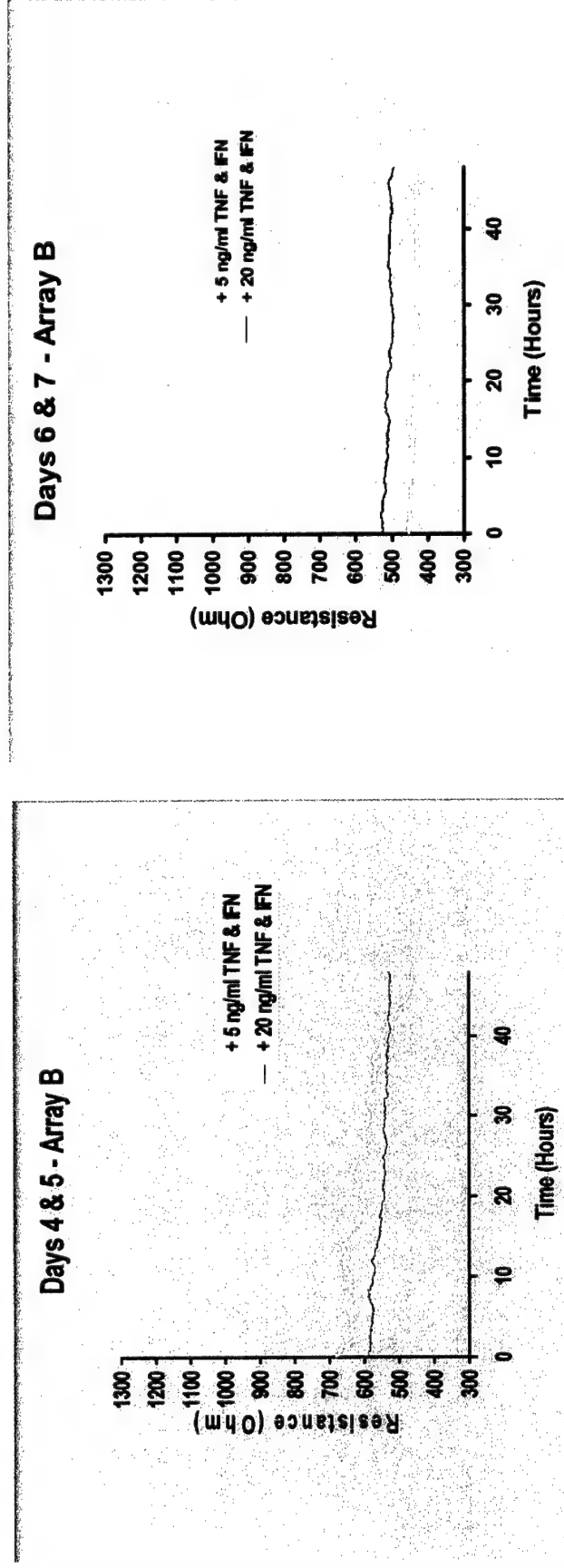
Figure 20

IL-1β + IFN-γ (Day 0 – Day 3)
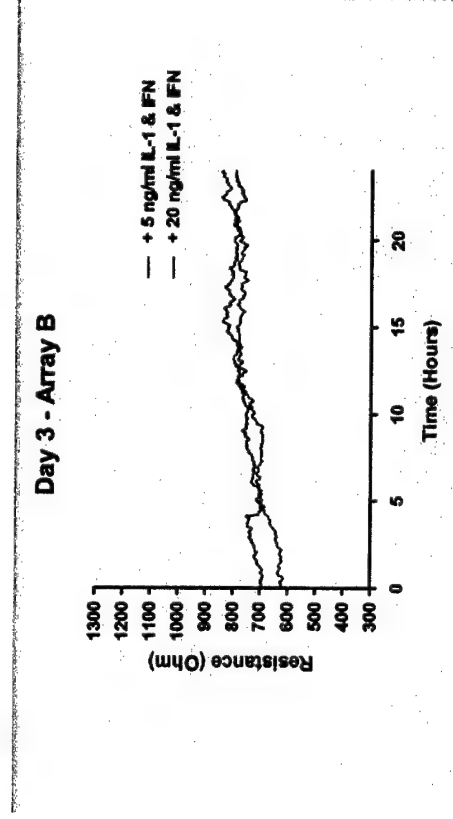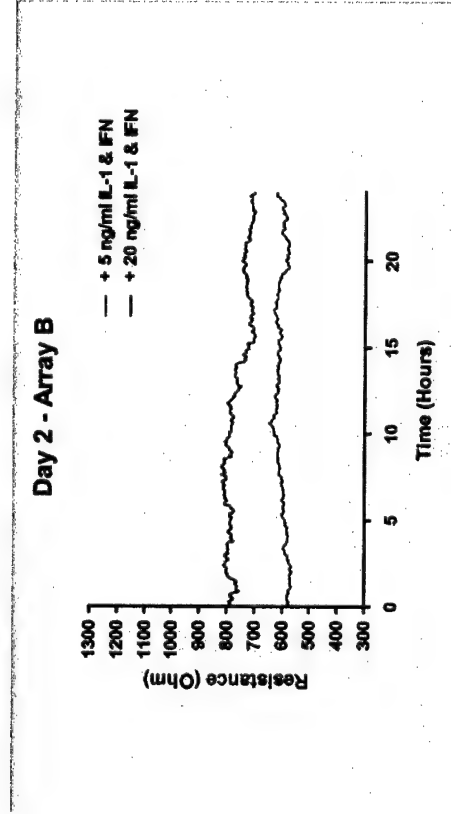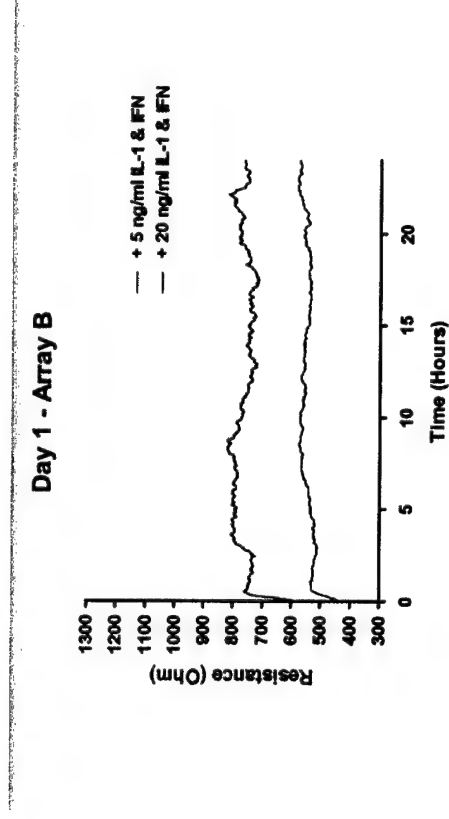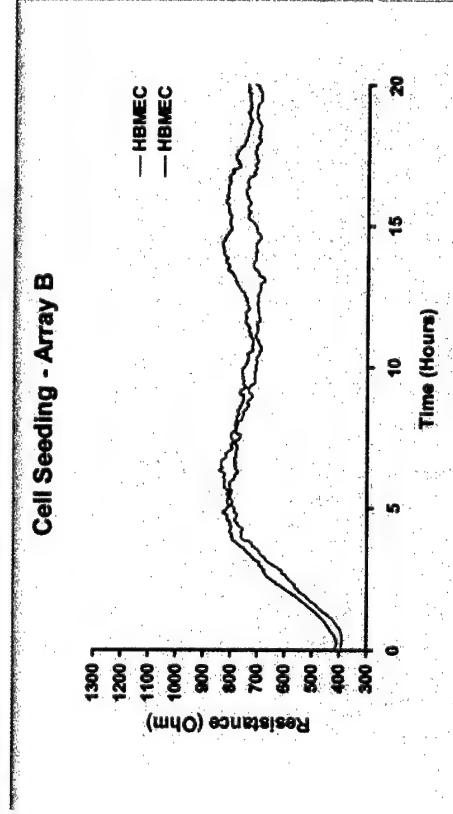
Figure 21

IL-1β + IFN-γ (Day 4 – Day 7)
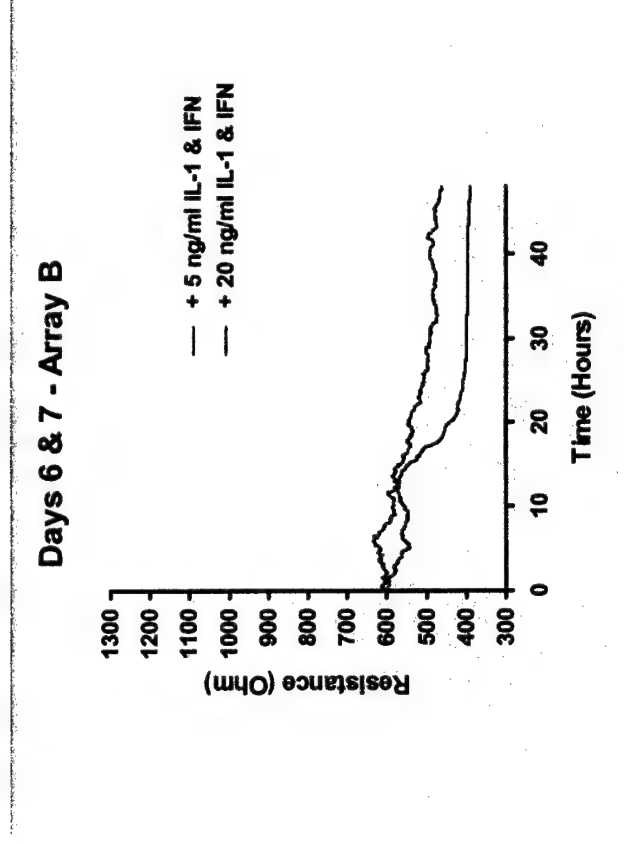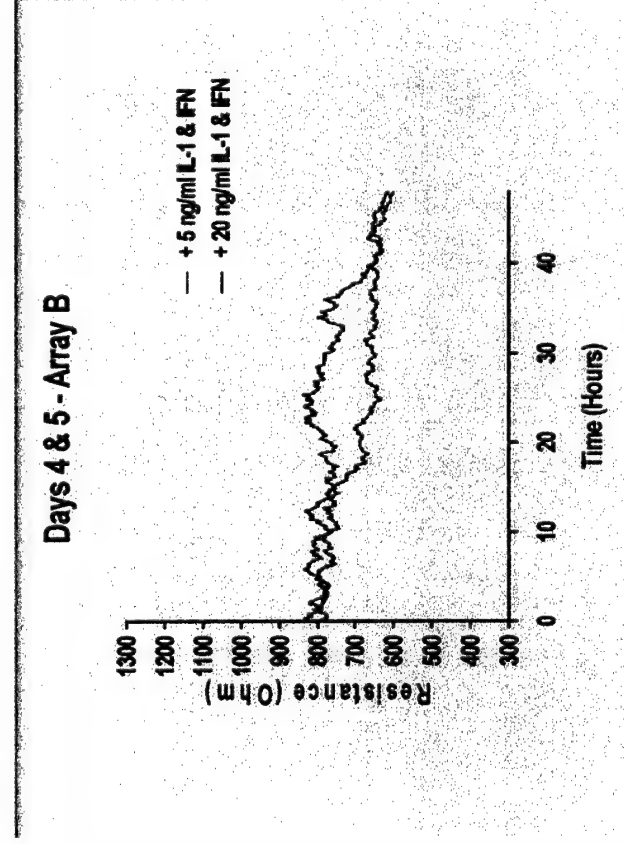
Figure 22

TNF-α + IL-1β + IFN-γ (Day 0 – Day 3)
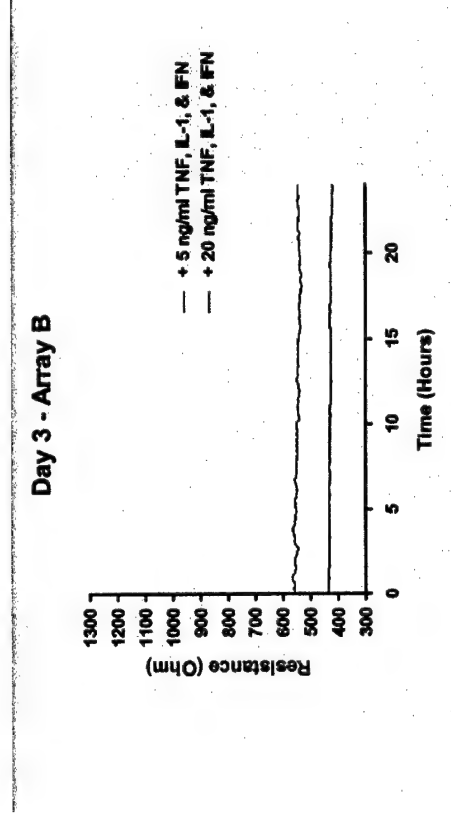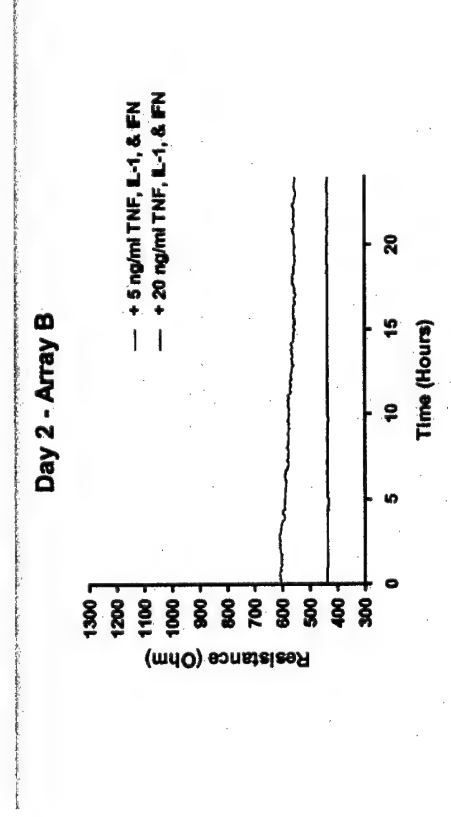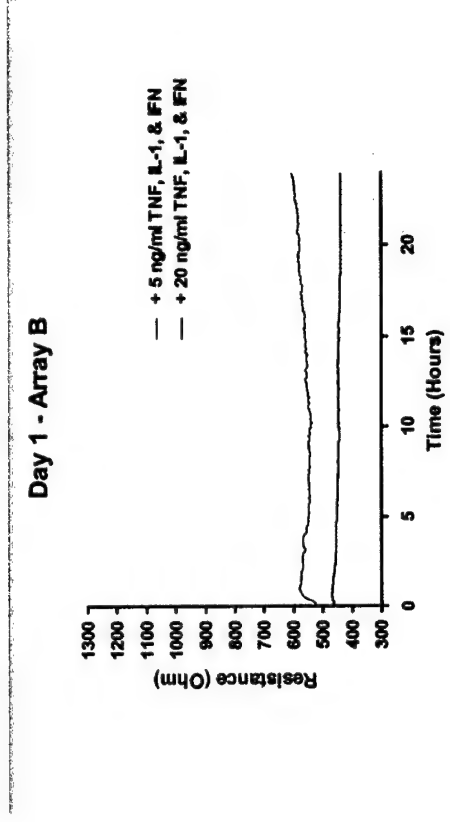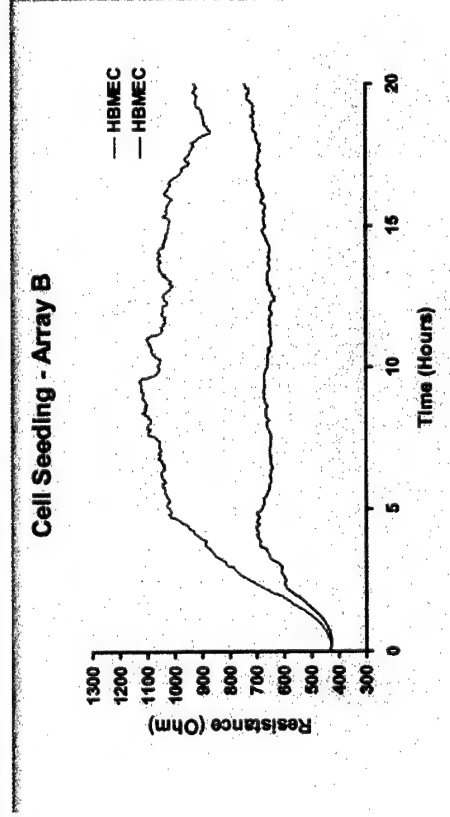
Figure 23

TNF-α + IL-1β + IFN-γ (Day 4 – Day 7)

Figure 24

GPNT, 0 - 48 Hours Post-Infection
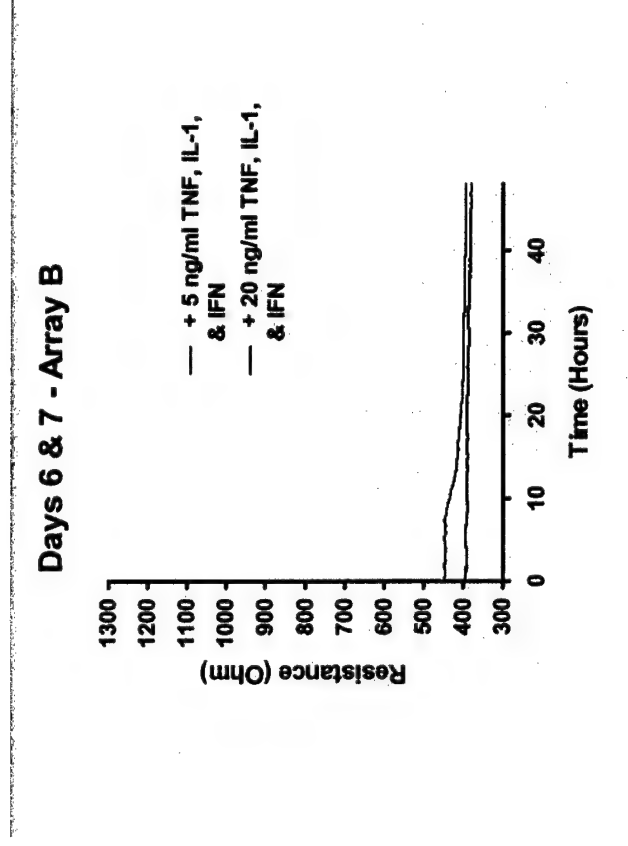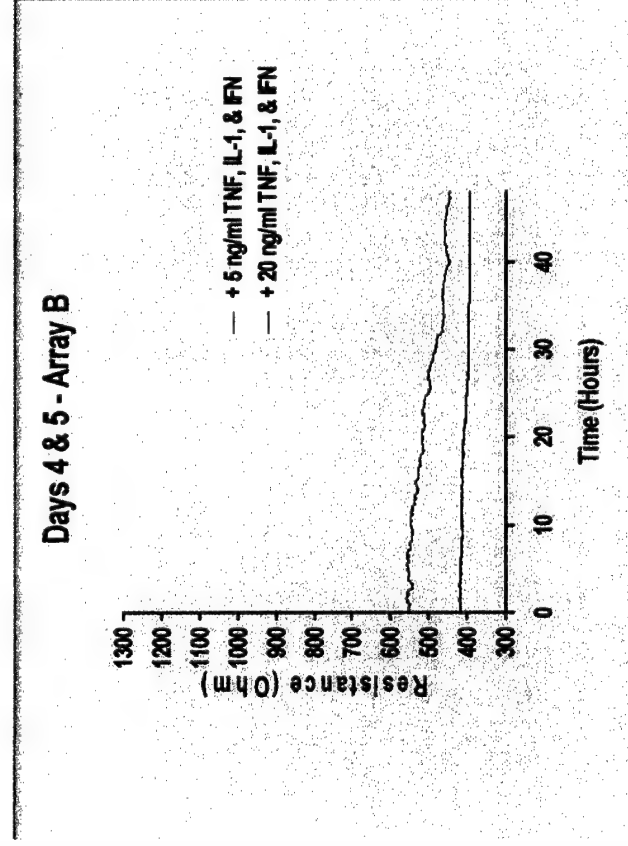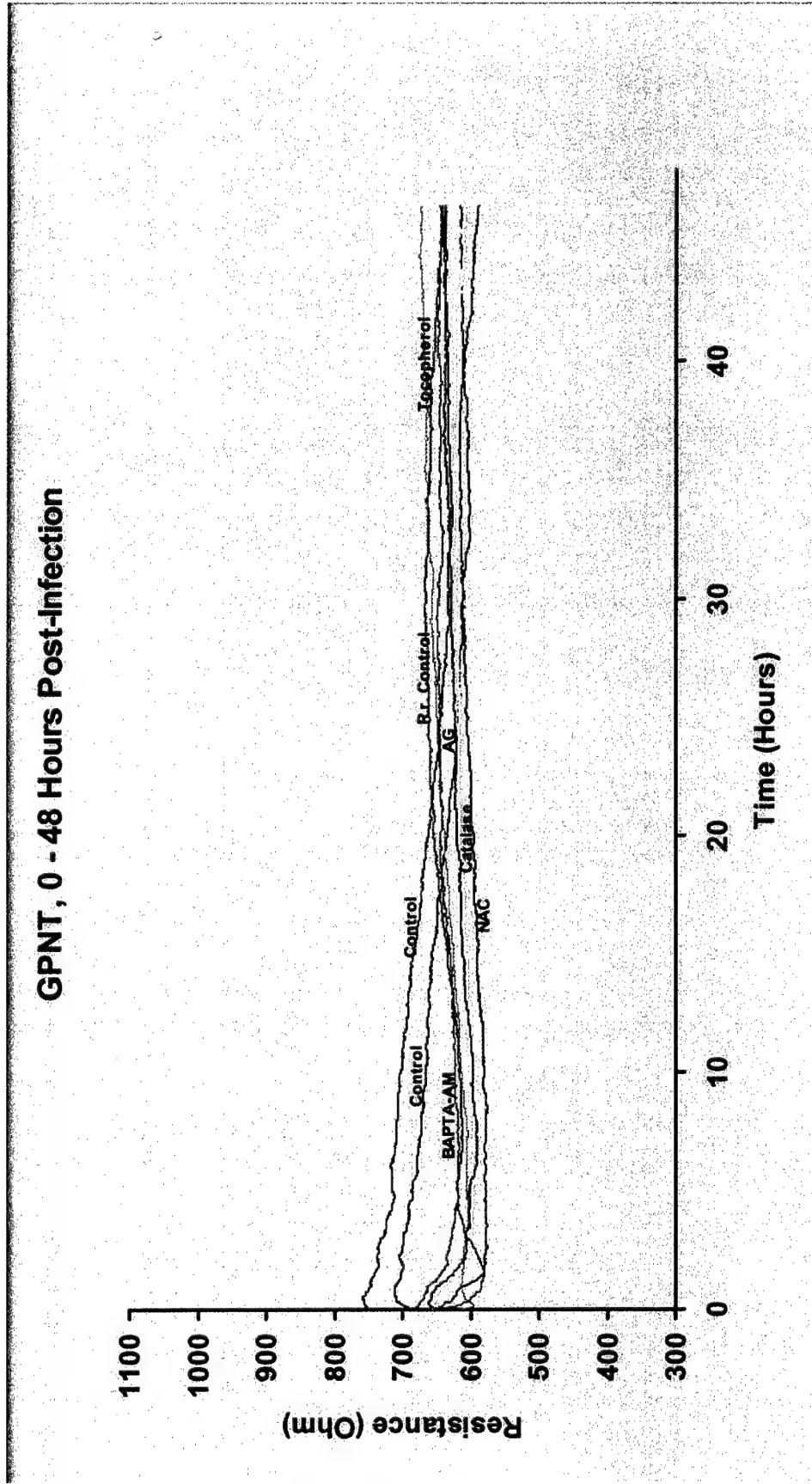
Figure 25

GPNT, 48 - 96 Hours Post-Infection

Figure 26

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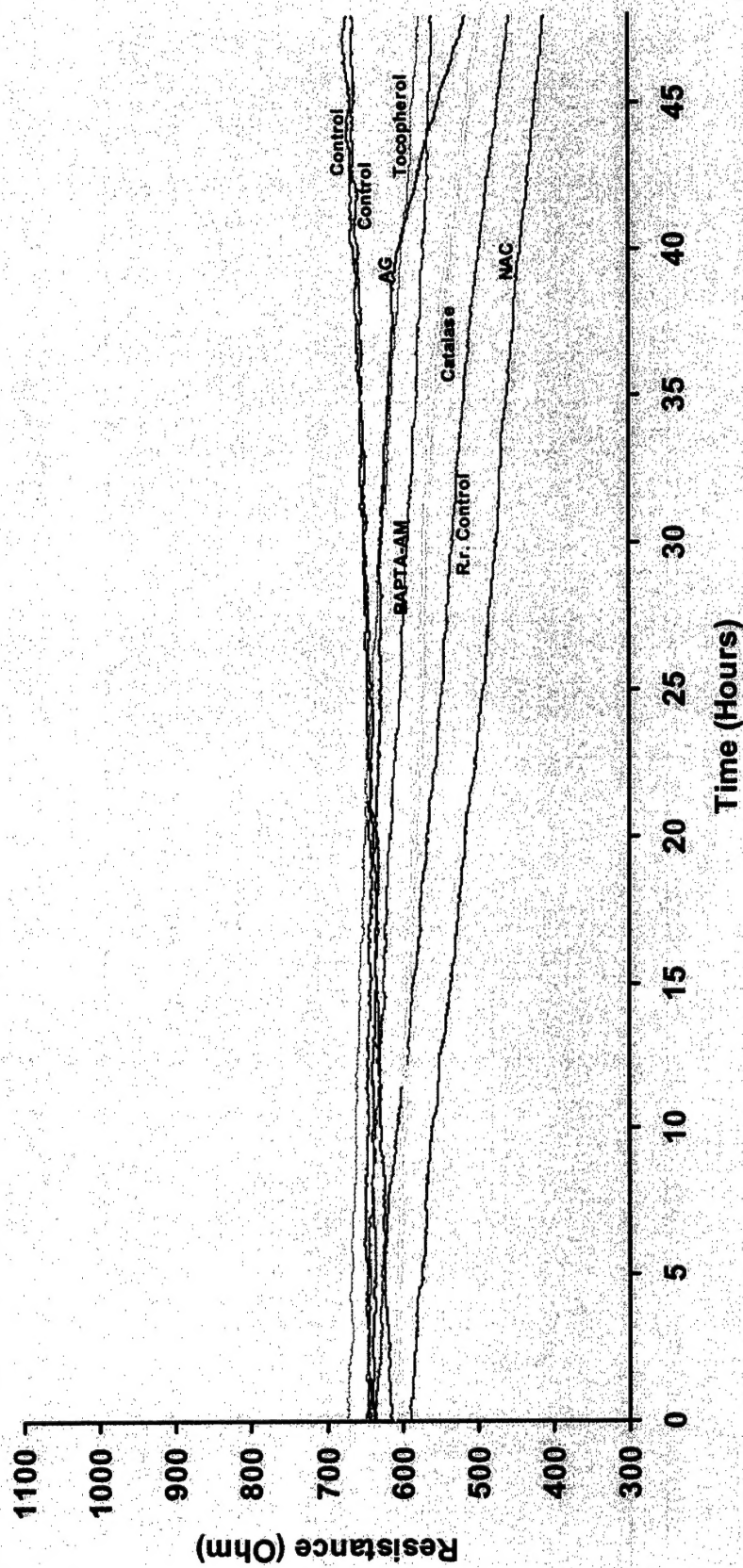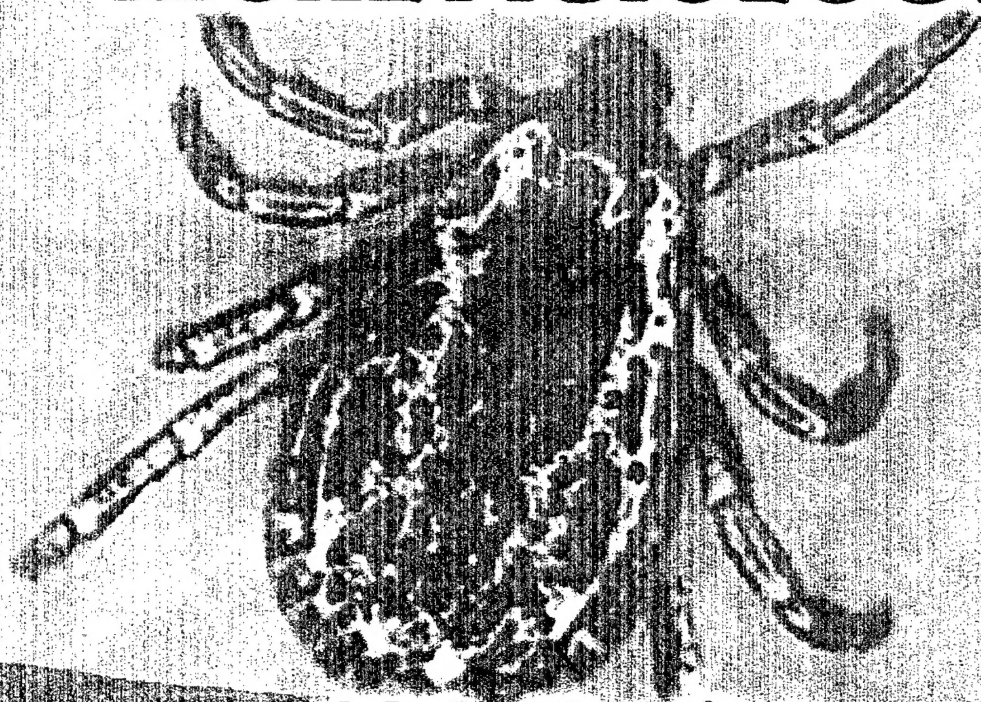
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Analysis of microvascular leakage in an *in vitro* model of the brain endothelial barrier by electric cell-substrate impedance sensing (ECIS).

Paul Koo and Juan P. Olano

University of Texas Medical Branch, Galveston, TX.

Increased microvascular permeability leading to cerebral and pulmonary edema is responsible for the morbidity and mortality seen in rickettsioses. The pathogenesis of increased microvascular permeability is unknown. We are in the process of developing an *in vitro* model of the brain endothelial barrier to study the molecular events leading to vascular leakage in acute rickettsioses. ECIS is a novel method to study transendothelial resistance (TER) across cell monolayers in real time on a continuous basis. A rat-derived microvascular endothelial cell line (RBE4) as well as human-derived primary cerebral endothelial cells (HBMEC) were grown on disposable arrays with gold electrodes. The monolayers were infected with *Rickettsia rickettsii*. Cytokines (IL-1-â, TNF-α and IFN-γ) were added to some of the infected and non-infected monolayers. Controls included non-infected monolayers without cytokine stimulation and array wells containing medium alone. Our experiments demonstrated that: a) Decreased TER (increased vascular permeability) was observed after 60 – 70 hours (RBE4) or 75 – 80 (HBMEC) of infection; b) The decrease in TER was directly proportional to the rickettsial inoculum; and c) The presence of cytokines potentiated a marked decrease in TER in the monolayers infected with rickettsiae. This *in vitro* model appears promising for the study of microvascular leakage in rickettsioses and other infectious diseases in general.

#67

Development of combinatorial peptide based libraries (adapteins) with rickettsicidal activity: Preliminary results

Paul Koo, Stanley Watowich, Robert Davey, Juan P. Olano*
University of Texas Medical Branch

Combinatorial peptide libraries (adaptein libraries) are a novel and powerful technique used to screen for compounds with potential microbicidal activity. Our system is based on delivery of genes within pantropic retroviruses coding for a scaffold protein (Venezuelan equine encephalitis virus capsid protein sub-domain fused to a peptide taken from the "tat" protein of HIV) into which small random peptide sequences (combinatorial 6-mer) are presented on an exposed loop (adaptein). The diversity of each library ranges between 2×10^7 and 2×10^8. Typical retroviral titers are approximately 107 pfu/ml. A rat brain derived endothelial cell line (RBE4) was infected with the retroviral vectors containing adaptein libraries and subsequently challenged with *Rickettsia rickettsii* at a MOI of 25. At 96 hours after the challenge, surviving cells still attached to the substratum were trypsinized, replated, and allowed to grow for 48 – 72 hours before being re-challenged with *R. rickettsii* (MOI = 15). Three more rounds of re-challenges (MOI = 10) were carried out. At the end of 5 rounds of challenges, 7 foci of putative rickettsia-resistant RBE4 cells were recovered. We are currently in the process of characterizing these cells at the molecular level to elucidate the mechanisms of resistance to rickettsial challenge.